UNIVERSIDAD PABLO DE OLAVIDE

FACULTAD DE CIENCIAS EXPERIMENTALES

DEPARTAMENTO DE BIOLOGÍA MOLECULAR E INGENIERÍA BIOQUÍMICA

ANÁLISIS DE LA DIVERSIDAD MICROBIANA ASOCIADA A LA FERMENTACIÓN DE ACEITUNAS VERDES DE MESA POR TÉCNICAS MOLECULARES DEPENDIENTES E INDEPENDIENTES DE CULTIVO

TESIS DOCTORAL

HELENA LUCENA PADRÓS

Sevilla, 2016

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Memoria de Tesis Doctoral que presenta la Licenciada

HELENA LUCENA PADRÓS

para optar al grado de Doctora en Biotecnología

Esta Tesis Doctoral ha sido realizada en el Departamento de Biotecnología de Alimentos del Instituto de la Grasa (CSIC)

Sevilla, 2016

El Dr. José Luis Ruiz Barba, Científico Titular del Consejo Superior de Investigaciones Científicas, adscrito al Instituto de la Grasa de Sevilla,

Y

el Dr. José Ignacio Ibeas Corcelles, Profesor Titular de la Universidad Pablo de Olavide de Sevilla, adscrito al Departamento de Biología Molecular e Ingeniería Bioquímica,

CERTIFICAN:

Que la Licenciada Helena Lucena Padrós ha realizado, bajo su dirección y tutela respectivamente, el trabajo titulado "Análisis de la diversidad microbiana asociada a la fermentación de aceitunas verdes de mesa por técnicas moleculares dependientes e independientes de cultivo", el cual reúne las condiciones de originalidad y calidad científica necesarias para optar al grado de Doctor por la Universidad Pablo de Olavide de Sevilla.

Y para que así conste, expedimos el presente certificado en Sevilla, a 1 de Abril de 2016.

Fdo.: **Fdo.:**

Dr. José Luis Ruiz Barba **Dr. José Ignacio Ibeas Corcelles**

Director de Tesis **Tutor de Tesis**

A mi madre y a mi hermana Irene

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Abreviaturas

ADN: ácido desoxirribonucléico

ANI: *average nucleotide identity* (identidad nucleotídica media)

ARN: ácido ribonucléico

ARNr: ácido ribonucléico ribosómico

ASEMESA: Asociación de Exportadores e Industriales de Aceitunas de Mesa

BAL: bacterias del ácido láctico

ºC: grados centígrados

CBD: Convenio sobre la Diversidad Biológica

COI: Consejo Oleícola Internacional

DGGE: *denaturing gradient gel electrophoresis* (electroforesis en gel con gradiente desnaturalizante**)**

ESYRCE: Encuesta sobre Superficie y Rendimientos de Cultivos

FISH: *fluorescence in situ hybridization* (hibridación fluorescente *in situ*)

G+C: guanina + citosina

ha: hectárea

HALAB: *halophilic and alkaliphilic lactic acid bacteria* (bacterias del ácido láctico halófilas y alcalófilas)

ICMSF: *International Commission on Microbiological Specifications for Foods* (Comisión Internacional de Especificaciones Microbiológicas de Alimentos)

ITSs: *internal transcribed spacer* (espacios transcritos internos)

MAGRAMA: Ministerio de Agricultura, Alimentación y Medio Ambiente

ML: *maximum likelihood* (máxima verosimilitud)

MP: *maximum parsimony* (máxima parsimonia)

NJ: *neighbor joining* (unión de vecinos)

pb: pares de bases

PCR: *polymerase chain reaction* (reacción en cadena de la polimerasa)

PFGE: *pulsed field gel electrophoresis* (electroforesis en gel de campo pulsado)

PIB: producto interior bruto

p/v: peso/volumen

RAPD-PCR: *random amplified polymorphic DNA-PCR* (amplificación aleatoria de ADN polimórfico mediante PCR)

rep-PCR: *repetitive element palindromic PCR* (amplificación por PCR de elementos palindrómicos repetitivos)

RFLP: *restriction fragment length polymorphism* (polimorfismos de longitud de fragmentos de restricción)

RT-PCR: *reverse transcription-PCR* (amplificación mediante PCR previa transcripción inversa)

SAU: superficie agraria útil

Tm: *melting temperature* (temperatura de fusión)

t: tonelada

UFC: unidades formadoras de colonia

v/v: volumen/volumen

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CAPÍTULO 1
INTRODUCCIÓN

1.1 La aceituna de mesa: definición, características, tipos y principales formas de elaboración

La Reglamentación Técnico-Sanitaria española y la Norma Cualitativa Unificada emitida por el Consejo Oleícola Internacional (COI) en 1980, denomina como aceituna de mesa al "fruto de variedades determinadas del olivo cultivado, sano, cogido en el estado de madurez adecuado y de calidad que, sometido a las preparaciones adecuadas, dé un producto de consumo y buena conservación como mercancía comercial".

El olivo (*Olea europaea L. var. sativa*), árbol del que procede la aceituna, constituye una de las plantas más antiguas domesticadas por el hombre (Zohary y Spiegel-Roy, 1975) y en la actualidad es el árbol frutal no-tropical más extensamente cultivado, con unos 900 millones de árboles productivos aproximadamente. La superficie cultivada dedicada al olivar se estima en torno a unos 10 millones de hectáreas y, si bien su hábitat se comprende entre las latitudes 30º y 45º tanto en el Hemisferio Norte como en el Hemisferio Sur (Barranco *et al*., 2004), es en la cuenca Mediterránea en donde se concentra el 98% del patrimonio olivarero (COI, 2014). No obstante, solo el 10% del olivar se destina a la producción de aceituna de mesa como alimento (Barranco, 2008), aunque las aceitunas de mesa representan el mayor volumen de vegetales fermentados en los países occidentales (Garrido-Fernández *et al*., 1997) y, muy especialmente, en los mediterráneos (Ercolini *et al*., 2006).

La aceituna es el único fruto en drupa no comestible en fresco, debido principalmente a la presencia de oleuropeína, compuesto fenólico glucósido responsable de su natural sabor amargo. Este fruto también se caracteriza por tener una baja concentración de azúcares (entre el 2,5 y el 6%) y un alto contenido en aceite (9-30%), si bien estos valores pueden variar en función del grado de madurez del fruto, la variedad, el origen geográfico y la técnica de cultivo.

Como drupa, este fruto se caracteriza por la presencia de tres capas:

- El exocarpio, o piel de la aceituna, es la capa más externa y fina, cuyo color varía desde el verde-amarillento hasta el rojizo-negro conforme el fruto avanza en su madurez.

- El mesocarpio carnoso, que se corresponde con la pulpa, es la parte comestible de la aceituna en la que se concentran los azúcares, la oleuropeína y el aceite. Si bien los niveles de los mismos evolucionan de forma inversa en función del grado de maduración. Así, mientras que en verde los niveles de azúcares y oleuropeína son máximos, conforme el fruto madura los niveles de aceite aumentan mientras disminuyen los anteriores.

- El endocarpio o hueso es único y está situado en el interior del fruto. Este contiene la semilla y suele representar entre el 13-30% del peso del fruto.

Por lo general, la clasificación de las variedades destinadas a la producción de aceite o de aceituna de mesa se realiza principalmente en base al contenido en aceite que alcanza el fruto maduro. Así, las variedades con contenidos grasos menores del 12% se destinan principalmente a aceitunas de mesa, mientras que aquellas con contenidos mayores del 20% se destinan a la producción de aceite de oliva (Ryan y Robards, 1998). El uso de los frutos de variedades con contenidos grasos intermedios varía en función de otros parámetros, tales como los económicos (es más cara la recolección de la aceituna de mesa), los culturales (tradición en una comarca) o el precio de mercado.

Sin embargo, en la práctica, la selección varietal para la producción de aceituna de mesa tiene que cumplir además con las siguientes características:

- Tamaño y forma adecuada: la aceituna se considera un fruto de pequeño tamaño, con unas dimensiones medias de 1 a 4 centímetros de longitud y de 0,6 a 2 centímetros de diámetro. Son los frutos con un peso igual o mayor a 4 gramos los más apropiados para la elaboración de aceituna de mesa. Por otro lado, las formas más esféricas y ovaladas tienen una mayor aceptación por el consumidor y facilitan su manejo industrial.

- Buena relación pulpa/hueso: una elevada relación pulpa/hueso aumenta el valor comercial de la misma y es necesaria para el deshuesado de las mismas.

- Pulpa delicada, sabrosa y firme, piel fina: sobre todo en frutos destinados al aderezo en verde deben ser resistentes a la acción de la lejía y la salmuera.

- Fácil separación de la pulpa y hueso: parámetro físico muy valorado por los consumidores y de gran importancia en el deshuesado.

Estas características quedan incluidas en la definición vigente del producto en la Norma Comercial del COI (2004) según la cual la aceituna de mesa es "el producto preparado a partir de frutos sanos de variedades de olivo cultivado (*Olea europaea L.*), elegidas por producir frutos cuyo volumen, forma, proporción de pulpa respecto al hueso, delicadeza de la pulpa, sabor, firmeza y facilidad para separarse del hueso los hacen particularmente aptos para la elaboración; sometido a tratamientos para eliminar el amargor natural y conservado mediante fermentación natural o tratamiento térmico, con o sin conservantes, y envasado con o sin líquido de gobierno."

A lo largo del tiempo se han desarrollado distintos métodos de elaboración de aceitunas de mesa que permiten no sólo su transformación en un fruto comestible sino en un alimento seguro y de gran calidad. Sin embargo, no todas las variedades son igual de aptas para las distintas formas de preparación, dando lugar a la obtención de productos finales muy diversos. Así, la Norma Comercial del COI (2004) clasifica las aceitunas de mesa según el grado de madurez en el que se recolectan y según su forma de preparación. Según el grado de madurez del fruto se distinguen hasta tres tipos: verdes, de color cambiante y negras. Según la forma de preparación, la Norma distingue entre aceitunas aderezadas, al natural, deshidratadas, negras por oxidación y otras especialidades. Sin embargo, desde el punto de vista comercial y por volumen de venta son tres los principales tipos de elaboración de aceitunas de mesa:

1) Aceitunas verdes aderezadas al estilo Español o Sevillano.
2) Aceitunas negras oxidadas o estilo Californiano.
3) Aceitunas negras al natural o estilo Griego.

Las primeras son aceitunas verdes que se someten a un tratamiento alcalino y una posterior fermentación láctica. Las segundas son aceitunas verdes que se someten a tratamiento alcalino en presencia de aire y sales de hierro, lo que las ennegrece, pero que no tienen una fermentación posterior y necesitan de la esterilización para conservarse. Las últimas son aceitunas maduras puestas directamente en salmuera hasta que desaparezca la mayor parte de su amargor y fermentadas principalmente por levaduras (Garrido-Fernández *et al*., 1997).

No obstante, desde un punto de vista biotecnológico, son en las aceitunas verdes aderezadas al estilo Español y las aceitunas negras al natural al estilo Griego las que tienen un mayor potencial debido a la importancia que tienen los microorganismos durante el proceso de elaboración para la obtención de un producto con características singulares.

1.2 Importancia del Sector del Aderezo de Aceitunas de Mesa en España

En España, la superficie de cultivo del olivo en producción es de 2.584.067 ha (ESYRCE, 2013), con más de 250 millones de árboles, lo que la convierte en el mayor olivar del mundo tanto en superficie como en número de olivos. No es de extrañar, por tanto, que España sea líder mundial en la producción y exportación de aceite de oliva y aceituna de mesa. Respecto al olivar dedicado a la aceituna de mesa, éste representa el 0,8% de la SAU (Superficie Agraria Útil) nacional y el 5,6% de la superficie total del olivar, con unas 144.904 ha, de las cuales 67.170 ha se corresponden con cultivares de doble actitud aceituna/aceite (MAGRAMA, 2014). Esta superficie está localizada mayoritariamente en Andalucía (83,67%) y Extremadura (14,13%). Dentro de estas Comunidades Autónomas, las provincias de Sevilla, Badajoz y Córdoba, seguidas de Cáceres y Málaga acaparan la mayoría del olivar destinado a aceituna de mesa. La producción nacional media de las últimas cinco campañas (2009/10-2013/14) ha sido de 176.500 t, lo que representa el 71% de la producción de la Unión Europea y el 21% de la producción mundial. Otros países de la cuenca mediterránea, junto con Argentina, son los principales competidores (Figura 1).

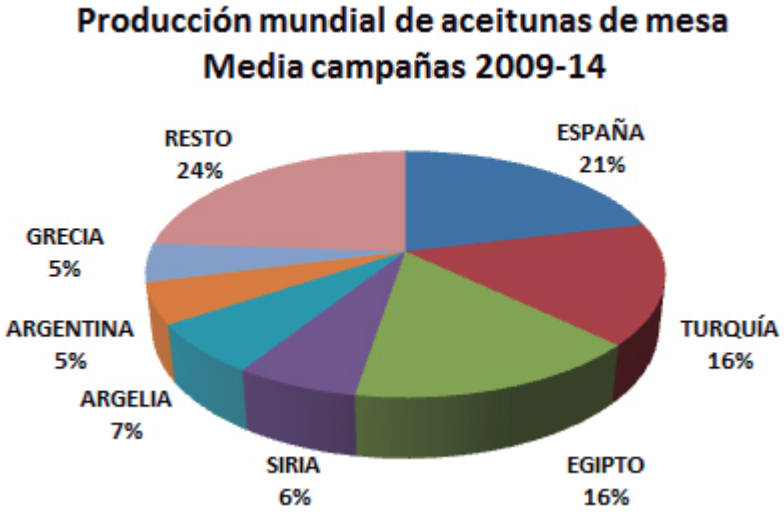

Figura 1. Producción mundial de aceitunas de mesa. Media de las campañas 2009/2014 (COI, 2014). Figura extraída de ASEMESA

Según datos del COI, la exportación media mundial de las últimas cinco campañas alcanzó las 880.000 t (peso neto escurrido). España, con 342.300 t, es el primer país exportador de aceituna de mesa, destinándose a este fin el 68% de la producción (media de los últimos seis años), y seguido a gran distancia por Grecia, Egipto, Turquía, Marruecos y Argentina, entre otros (Figura 2).

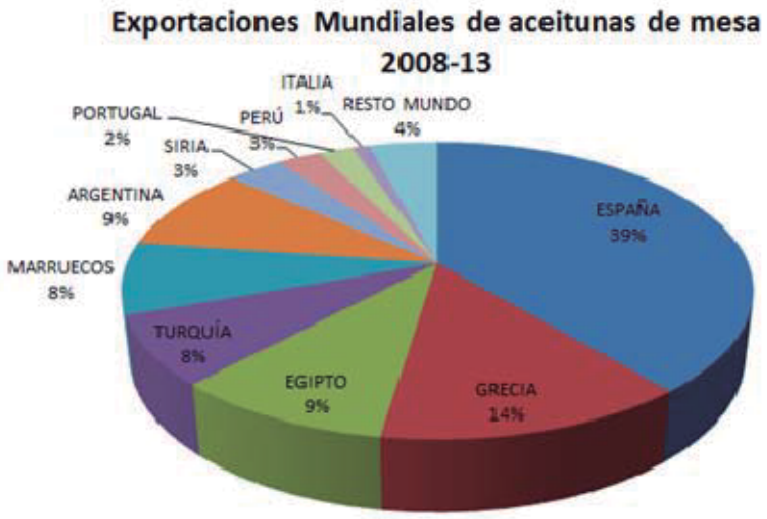

Figura 2. Exportación mundial de aceitunas de mesa. Media de las campañas 2008/2013 (COI, 2014). Figura extraída de ASEMESA (2014)

La aceituna española se exporta a 120 países distribuidos por los cinco continentes. Los Estados Unidos, Italia y Rusia son los mayores importadores de aceituna de mesa española. Además, España es el cuarto país con un mayor consumo de aceituna de mesa, por detrás de Turquía, Egipto y Estados Unidos, según datos del COI de las cinco últimas campañas. La media de consumo mundial es de 2.482.700 t anuales (COI, 2015). Por otro lado, el Sector del Aderezo de Aceitunas de Mesa goza de gran relevancia en el conjunto de la industria agroalimentaria nacional, tanto por el número de empleos que genera como por los beneficios económicos obtenidos. Así, son de destacar los más de 8.000 empleos directos y los seis millones de jornales por campaña generados, a los que hay que añadir los creados por las empresas y fábricas auxiliares como las de vidrio, hojalata, maquinaria, transportes, etc. Esto supone el 27% del empleo generado por el Sector Nacional de Conservas y Preparados de Productos Vegetales, participando con un 22% en el valor nacional de este sector y aportando al PIB alrededor de 1.000 millones de euros (ASEMESA, 2014).

En cuanto a la distribución de las empresas dedicadas a la elaboración de aceituna de mesa en nuestro país, éstas se hallan en concordancia con las zonas de máxima producción. Así, de las 491 industrias dedicadas a este sector, 280 se encuentran en Andalucía y, a su vez, la mayoría localizadas en la provincia de Sevilla.

1.3 Elaboración industrial de aceitunas verdes aderezadas al estilo Español

Las aceitunas verdes elaboradas al estilo Español constituye uno de los principales tipos de preparación de aceitunas de mesa, abarcando aproximadamente el 55-60% de la producción mundial, según campañas (Garrido Fernández *et al.,* 1997; Panagou *et al*., 2008). En nuestro país, el 70% de la producción de aceituna de mesa se destina para su consumo como aceituna verde (MAGRAMA, 2015). Este método de elaboración es también el de mayor importancia económica en nuestro país, como demuestra el hecho de que el 78% de las industrias del sector se dedican sólo a esta preparación (Estrada, 2011). El diagrama general del proceso de aderezo de la aceituna verde al estilo Español se muestra en la Figura 3.

Figura 3. Diagrama de flujo del proceso de aderezo de la aceituna verde al estilo Español. (Adaptado de Garrido-Fernández *et al.,* 1997).

Este procedimiento se caracteriza por la recolección en verde de las aceitunas, lo que popularmente se conoce como "verdeo" y que se suele extender durante los meses de septiembre a noviembre en cada campaña dependiendo del lugar, la climatología y las variedades de aceitunas, principalmente. Estas aceitunas verdes, tras un proceso de escogido para eliminar las no aptas por color o diversos daños, son sometidas a un tratamiento alcalino suave con hidróxido de sodio ("sosa" o "lejía", en el argot propio del sector) a una concentración variable entre el 1,8 y el 3,5% (v/v). Esta operación, denominada “cocido”, se realiza para eliminar el amargor natural de los frutos, al tiempo que se neutralizan una serie de compuestos fenólicos de carácter inhibidor, que impedirían una posterior fermentación ácido láctica, y se permeabiliza la epidermis de los frutos (Ruiz-Barba y Jiménez-Díaz, 1989; Ruiz-Barba *et al.,* 1990, 1991a, 1991b y 1993; Medina, 2008). Este tratamiento termina habitualmente cuando la lejía penetra entre 2/3 y 3/4 partes de la distancia de la piel al hueso, un proceso que dura entre 6 y 10 horas según, principalmente, la concentración de hidróxido de sodio empleada y la variedad de aceituna. Posteriormente, tras una serie de lavados con agua para eliminar el exceso de lejía y los compuestos fenólicos extraídos, las aceitunas se cubren con una salmuera. Esta salmuera está elaborada con sal común, apta para consumo humano, disuelta en agua potable al 10-12% (p/v). La concentración de esta salmuera quedará en torno a un 5-7% tras el equilibrio, alcanzado en aproximadamente una semana, y es aquí donde se va a desarrollar una fermentación espontánea del tipo ácido láctica, tomando como sustrato los azúcares que pasan desde la pulpa de las aceitunas a la salmuera.

En la fermentación de aceitunas verdes de mesa se han identificado hasta tres fases sucesivas, atendiendo al tipo de microorganismos que están presentes y a las características físicas y químicas (principalmente valores de pH y acidez libre) de la salmuera (Garrido-Fernández *et al.,* 1995) [ver apartado 1.4]. Si se desarrolla correctamente, esta fermentación es llevada a cabo principalmente por bacterias del ácido láctico (BAL), más concretamente por cepas de la especie *Lactobacillus pentosus* (De Castro et al., 2002; Rejano et al., 2010; Ruiz-Barba y Jiménez-Díaz, 2012).

El proceso termina cuando se han consumido todos los azúcares de la salmuera y se han alcanzado valores de pH y acidez libre apropiados para que el producto adquiera las características organolépticas deseadas y se pueda conservar adecuadamente. Estos valores se encuentran entre 3,8 y 4,2 para el pH, y 0,8 y 1,2% para la acidez libre (expresada como ácido láctico) (Fernández-Díez, 1983). Finalmente, los frutos ya fermentados son seleccionados,

clasificados y, tras posibles operaciones de acondicionamiento como el deshuesado y relleno, quedan listos para envasar. El producto envasado suele ser pasterizado, aunque no siempre es así, ya que las condiciones de pH, acidez libre y concentración de sal garantizan en la mayoría de los casos una buena conservación.

Por lo general, el cocido, los lavados y la puesta en salmuera tienen lugar en instalaciones del interior de la planta de aderezo, habitualmente en depósitos aéreos ("cocederas") con una capacidad para procesar entre 10.000 y 15.000 kg de aceitunas. Estas operaciones suelen tener una duración total máxima de 24 horas. Finalmente, los frutos inmersos en la salmuera se trasvasan mediante sistemas de tuberías a los fermentadores. Estos fermentadores son, en general, depósitos esféricos o cilíndricos construidos en poliéster reforzado con fibra de vidrio. Su capacidad, como la de las cocederas, varía para contener entre 10 y 15 t de frutos. Los fermentadores normalmente se localizan en el exterior de las instalaciones, en lo que se denominan "patios", y suelen estar enterrados para evitar las fluctuaciones térmicas (Rejano *et al*., 1977; Borbolla y Alcalá y Rejano, 1979, 1981). En ellos tiene lugar la fermentación que, si todo va bien, es del tipo ácido láctica y que suele durar entre dos y tres meses, aunque es variable atendiendo a factores tales como la temperatura ambiente y otros condicionantes (Fernández-Díez *et al*., 1985).

Por variedades, las aceitunas destinadas a "verdeo" más difundidas en nuestro país son:

- Manzanilla fina sevillana: es la principal variedad empleada en la elaboración de aceitunas verdes de mesa al estilo Español y la de mayor tradición. Su cultivo, originario de la provincia de Sevilla, presenta una gran internacionalización, hallándose también en países como Portugal, Israel, Australia, Argentina y Estados Unidos. De gran calidad para el aderezo, por las características organolépticas que adquiere durante el proceso, presenta un tamaño medio (calibre de 200/280 frutos/kg), forma aproximadamente esférica, sin pezón, con una elevada relación pulpa/hueso y fácil desprendimiento del hueso. Su contenido en aceite también permite su empleo en almazara, lo que sucede principalmente cuando hay un exceso de producción.

- Carrasqueña: variedad Manzanilla sevillana sobre-injertada en la variedad local Verdial de Badajoz, que se cultiva al norte de la provincia de Badajoz y en Cáceres.

- Gordal: se cultiva en la provincia de Sevilla y es una variedad muy apreciada a nivel mundial, en especial en Estados Unidos. De gran tamaño, con un calibre medio de

100/120 frutos/kg, alcanzan un peso medio de 12,5 gramos por fruto. De aspecto acorazonado y de color verde con pintas blancas. Debido a su bajo contenido en aceite solo se destina a la producción de aceituna de mesa.

- Hojiblanca: se cultiva principalmente al sur de las provincias de Córdoba, Málaga, Sevilla y Granada, de maduración tardía. Variedad de doble aptitud, aceituna de mesa y aceite, también es muy apreciada en la preparación de aceitunas negras al estilo californiano por la firmeza de su pulpa, característica que requiere este tipo de elaboración. De forma alargada y pequeño tamaño. En la actualidad, es la variedad que mayor crecimiento ha tenido en el mercado dada la resistencia de los frutos al molestado, lo que permite la recolección mecánica.

Otros cultivares de nuestro país dedicados a la producción de aceitunas verdes de mesa al estilo Español, aunque en menor proporción, son Cacereña, Morona, Aloreña, Verdial, etc.

Las condiciones de las operaciones iniciales, principalmente del cocido y lavado de las aceitunas, se modifican en función de la variedad empleada debido a las propiedades de cada cultivar (tamaño, textura, amargor...) (Montaño *et al*., 2003). Por ejemplo, los frutos de la variedad Manzanilla, de recolección manual y de piel bastante delicada, frecuentemente se transportan empleando lejías diluidas o se les aplica un "reposo" durante unas 48 h previo al cocido para evitar el "despellejado". Por otra parte, las condiciones físico-químicas alcanzadas al final de la fermentación también dependen de la variedad empleada. Así, por ejemplo, la variedad Hojiblanca alcanza niveles más altos de pH y acidez combinada que las variedades Manzanilla y Gordal. Por otra parte, la composición química de las salmueras ya fermentadas, respecto a sus componentes principales tales como el ácido láctico, etanol o ácido fórmico, que presenta la variedad Manzanilla son muy diferentes de los observados para la variedad Gordal, por ejemplo (Montaño *et al*., 2003).

1.4 Microbiología y fases características de las fermentaciones de aceitunas verdes aderezadas al estilo Español

Pese a que el proceso tecnológico de fermentación de aceitunas de mesa al estilo Español está bien definido, al tratarse de una fermentación abierta, en la cual el material de partida no puede ser esterilizado, y a las características de los fermentadores, esta fermentación está sujeta a una cierta variabilidad. Esta variabilidad tiene su expresión en la sucesión ecológica

de microorganismos que intervienen en el proceso y de los que dependen las propiedades del producto final. Aún en la actualidad, la fermentación de la gran mayoría de las aceitunas de mesa se inicia de forma espontánea. El seguimiento del proceso se hace basándose, casi exclusivamente, en análisis fisicoquímicos, en especial en los valores de pH y acidez libre.

Tradicionalmente, ya desde los estudios realizados por Vaughn *et al*. (1943) y, posteriormente, por Borbolla y Alcalá (1956) y Borbolla y Alcalá y González Cancho (1975), en la fermentación de aceitunas de mesa al estilo Español se identifican tres fases distintas y sucesivas en función de las la poblaciones microbianas predominantes y de las características físico-químicas que van adquiriendo las salmueras.

La primera fase de la fermentación comienza con la colocación de los frutos, ya tratados con hidróxido de sodio, en salmuera. El pH inicial es superior a 10 unidades y la concentración de sal suele ser del 10% (p/v). Ya al final de la primera semana, la concentración de sal alcanza un equilibrio con la pulpa de las aceitunas, quedando en el rango del 5-7%, aproximadamente. Esta primera fase termina cuando el valor de pH se acerca a las 6 unidades. Microbiológicamente, esta fase se caracteriza por la presencia de diversos grupos de microorganismos álcali-tolerantes no relacionados entre sí: bacilos Gram-negativos (principalmente *Enterobactereaceae*), bacterias Gram-positivas esporuladas, bacterias cocáceas del ácido láctico (principalmente *Leuconostoc*, *Aerococcus* y *Enterococcus*), levaduras y mohos (Fernández-Díez *et al.,* 1985). Al final de esta fase, la concentración de bacterias Gram-negativas desciende hasta virtualmente desaparecer, mientras que las especies del género *Lactobacillus* ya se empiezan a aislar. Esta fase suele tener habitualmente una duración entre 5 y 15 días, aproximadamente, dependiendo de una serie de factores tales como la temperatura, el inóculo microbiano inicial (espontáneo o no), el número e intensidad de los lavados tras el tratamiento alcalino, etc.

La segunda fase de la fermentación está dominada por BAL, principalmente especies del género *Lactobacillus* y, más concretamente, cepas de la especie *L. pentosus* (de Castro *et al*., 2002; Rejano *et al*., 2010; Ruiz-Barba y Jiménez-Díaz, 2012). También es característica la presencia de diversas especies de levaduras (Arroyo-López *et al.,* 2006; Bautista-Gallego *et al*., 2011). El crecimiento exponencial de las BAL hace bajar los valores de pH hasta 4,0-4,5, y subir los de acidez libre, principalmente en forma de ácido láctico, hasta 0,25-0,5%. Esta fase suele tener una duración de un mes, aproximadamente, dependiendo de parámetros similares a los expuestos más arriba para la primera fase.

Durante la tercera fase de la fermentación, el crecimiento de las BAL se hace estacionario, los azúcares se empiezan a agotar, los valores de pH llegan a estar por debajo de 4,0 unidades y la acidez libre suele alcanzar valores entorno al 0,8-1,2%. Esta fase suele durar de uno a dos meses más, completándose así el proceso de fermentación de aceitunas verdes de mesa. Los valores de pH, acidez libre y concentración de sal alcanzados en esta fase suelen ser suficientes para garantizar la conservación de los frutos así procesados hasta su clasificación y posterior envasado.

Por otra parte, algunos autores han llegado a identificar una cuarta fase, no deseada, en la fermentación de este tipo de aceitunas de mesa. Esta fase se puede manifestar cuando las condiciones finales alcanzadas hacen posible el crecimiento de determinados microorganismos alterantes. Así, esta cuarta fase estaría caracterizada por una elevación de los valores de pH y una reducción de los valores de acidez libre debido a la actividad metabólica de ciertas especies bacterianas, principalmente del género *Propionibacterium*, que se encuentran como contaminantes en las propias salmueras de fermentación (Fernández-Díez *et al.,* 1985; Garrido-Fernández *et al.,* 1995; Arroyo-López, 2006). En estas condiciones, pueden aparecer otras bacterias que alteran aún más el producto, e incluso ser patógenas, como pueden ser especies del género *Clostridium.* Aparte de las consideraciones respecto a la salubridad del producto final, todo ello ocasiona pérdidas a la industria debidas a la falta de calidad del producto y a los tratamientos adicionales que requiere el acondicionamiento de la producción defectuosa.

De lo anterior se desprende que existe la necesidad de dirigir y controlar la fermentación de aceitunas de mesa. Al tratarse de una fermentación natural y generalmente espontánea, la acción de la diversa microbiota autóctona tiene un papel fundamental en la gran variabilidad de las propiedades organolépticas y la conservación del producto final, así como en el curso de la fermentación y, por tanto, en el rendimiento económico de la producción. Por todo ello se ha propuesto el uso de cultivos iniciadores como uno de los mayores avances biotecnológicos que se pueden llevar a cabo para este tipo de fermentación (Ruiz-Barba *et al.,* 1994). La selección de un cultivo iniciador es un proceso complejo que precisa de un conocimiento preciso de la ecología, la diversidad microbiana y su evolución durante la fermentación de aceitunas verdes de mesa. Además, la correcta elección de un cultivo iniciador ha de realizarse atendiendo a dos principios básicos: que realice la actividad metabólica deseada y que sea capaz de competir eficientemente en su medio (Buckenhüskes,

1993). En la actualidad, los cultivos iniciadores para la elaboración de aceitunas de mesa disponibles en el mercado son pocos y se aplican indiscriminadamente a todo tipo de variedades de aceitunas, localizaciones geográficas, climatología, condiciones de procesamiento específicas, etc. Por lo tanto, el aislamiento, identificación y conservación de la microbiota autóctona presente en las fermentaciones de aceitunas verdes de mesa, especialmente la procedente de zonas geográficas con altas producciones al ser la mejor adaptada a este proceso, será la mejor forma de garantizar a largo plazo la calidad y la conservación de las características propias del producto final.

Capítulo 2
Objetivos

El objetivo primario de esta Tesis Doctoral es caracterizar la diversidad microbiana asociada a las fermentaciones de aceitunas verdes de mesa al estilo Español. Estudios anteriores de carácter amplio sobre la microbiota característica de este tipo de elaboración son escasos y, sobre todo, anticuados tanto por las técnicas de muestreo como por los criterios de clasificación microbiana empleados. Por otra parte, este producto se basa en una forma de preparación muy antigua, cuya industrialización comenzó a finales del siglo XIX en la provincia de Sevilla (Estrada, 2011). Desde entonces hasta hoy, como se señala en el capítulo de Introducción, el proceso de elaboración ha sufrido considerables cambios que, a nuestro entender, deben haber afectado sustancialmente al proceso fermentativo. Así, estos cambios tienen que ver, por ejemplo, con la mecanización, el aumento progresivo en el tamaño de los fermentadores, el acortamiento del tiempo en las operaciones previas a la fermentación, la aceleración de la misma mediante correcciones en las condiciones físico-químicas (pH y acidez, principalmente), la incorporación de medidas para reducir los vertidos de efluentes líquidos, tales como la reutilización de las lejías o la adopción de protocolos de buenas prácticas higiénicas de fabricación (Santos-Siles, 1999). Todo ello, por otra parte, ha contribuido a evitar la aparición de alteraciones en el producto o de enfermedades de transmisión alimentaria (Estrada, 2011). Resulta conveniente, por tanto, actualizar y discernir los posibles cambios en la diversidad y dinamismo de las distintas poblaciones microbianas asociadas a las fases de fermentación previamente descritas.

Un segundo objetivo es evaluar distintas metodologías, clásicas y modernas, para describir y abordar con un enfoque ecológico el estudio de la composición, estructura y dinamismo de las comunidades microbianas inherentes a las fermentaciones de aceitunas. Así, se ha combinado el empleo de técnicas moleculares dependientes e independientes de cultivo. La utilización de técnicas moleculares, basadas en el ADN para la identificación de los aislados, en sustitución a la antigua metodología basada en el fenotipo de los mismos (morfología, fisiología y perfil bioquímico), permite describir la biodiversidad de este ecosistema de una forma más rápida, precisa y económica. De esta manera, se podrá revisar más fácilmente el potencial de este hábitat para el hallazgo de nuevas especies o taxones microbianos, lo que constituye en sí mismo otro de los objetivos del presente trabajo.

Finalmente, los trabajos realizados permitirán obtener una amplia colección de aquellos microorganismos (bacterias y levaduras) que colonizan la fermentación de este tipo de aceitunas de mesa de forma secuencial en cada una de las distintas fases definidas para esta

elaboración. Estos microorganismos serán debidamente identificados taxonómicamente a nivel de especie y de cepa, a la par que se podrán asociar a las principales características fisicoquímicas de las salmueras de las que fueron aislados.

Para este estudio se han elegido dos industrias de la provincia de Sevilla con una larga tradición en la elaboración de aceitunas de mesa y, más concretamente, aceitunas verdes aderezadas al estilo Español. Estas dos industrias pueden ser ambas consideradas "de gran tamaño", dadas sus producciones anuales (4.000-8.000 t), y en ellas la fermentación está muy mediatizada por factores industrializados del procesamiento así como por toda una serie de intervenciones tecnológicas (correcciones de pH, acidez libre, sal, etc.) que dirigen, pero a veces también restringen, el proceso fermentativo natural.

En resumen, el propósito último de este trabajo es describir, rescatar y conservar la diversidad microbiana vinculada a las fermentaciones de aceitunas verdes de mesa. Con ello se mejorará nuestro entendimiento sobre las mismas con el objetivo de obtener productos seguros con características tipificadas. La finalidad de este proyecto y su importancia se sustentan también en la actual preocupación por la calidad alimentaria y la posible pérdida de diversidad microbiana ocasionada por la homogenización del proceso tecnológico a escala industrial y al emergente uso de inóculos comerciales para la fermentación de aceitunas verdes de mesa.

2.1 Sub-objetivos y publicaciones en las que se abordan

2.1.1 Estudio de la diversidad microbiana de la fermentación de aceitunas verdes de mesa mediante técnicas moleculares dependientes de cultivo

- Lucena-Padrós, H., Caballero-Guerrero, B., Maldonado-Barragán, A. y Ruiz-Barba, J.L. (2014b). **Microbial diversity and dynamics of Spanish-style green table-olive fermentations in large manufacturing companies through culture-dependent techniques.** *Food Microbiology* 42, 154-165.

- Lucena-Padrós, H., Caballero-Guerrero, B., Maldonado-Barragán, A. y Ruiz-Barba, J.L. (2014d). **Genetic diversity and dynamics of bacterial and yeast strains associated to Spanish-style green table-olive fermentations in large manufacturing companies.** *International Journal of Food Microbiology* 190, 72-78.

- Lucena-Padrós, H. y Ruiz-Barba, J.L. (2016). **Diversity and enumeration of halophilic and alkaliphilic b acteria in Spanish-style g reen table-o live fermentations.** *Food Microbiology* 53, 53-62.

2.1.2 Estudio de la diversidad microbiana asociada a la fermentación de aceitunas verdes de mesa mediante técnicas moleculares independientes de cultivo

- Lucena-Padrós, H., Jiménez, E., Maldonado-Barragán, A., Rodríguez, J.M. y Ruiz-Barba, J.L. (2015b). **PCR-DGGE assessment of the ba cterial diversity in Spanish-style green table-o live fermentatio ns.** *International Journal of Food Microbiology* 205, 47–53.

2.1.3 Aislamiento y descripción taxonómica de nuevas especies microbianas procedentes de fermentaciones de aceitunas verdes de mesa

- Lucena-Padrós, H., González, J.M., Caballero-Guerrero, B., Ruiz-Barba, J.L. y Maldonado-Barragán, A. (2014a). ***Enterococcus olivae* sp. nov. , isolated from Spanish-style green o live fermentations.** *International Journal of Systematic and Evolutionary Microbiology* 64, 2534–2539.

- Lucena-Padrós, H., González, J.M., Caballero-Guerrero, B., Ruiz-Barba, J.L. y Maldonado-Barragán, A. (2014c). ***Propionibacterium olivae* sp. nov . and *Propionibacterium damnosum* sp. nov ., isolated from spoiled packaged Spanish-style green olives.** *International Journal of Systematic and Evolutionary Microbiology* 64, 2980-2985.

- Lucena-Padrós, H., González, J.M., Caballero-Guerrero, ., Ruiz-Barba, J.L. y Maldonado-Barragán, A. (2015a). ***Vibrio olivae* sp. nov., isolated from Spanish-style green-olive fermen tations.** *International Journal of Systematic and Evolutionary Microbiology* 65, 1895-1901.

Capítulo 3
Marco teórico

3.1 Diversidad microbiana: concepto y razones para su estudio en la industria alimentaria

El Convenio sobre la Diversidad Biológica (CBD) de Río de Janeiro en 1992 define la biodiversidad como "la variabilidad de los organismos vivos procedentes de cualquier fuente, incluyendo los ecosistemas terrestres, marinos y otros ecosistemas acuáticos y los complejos ecológicos de los que forman parte". La biodiversidad también ha sido definida de acuerdo a la teoría de la información como la cantidad y distribución de la información dentro de un ensamblaje o comunidad (Torsvik *et al*., 1998). Por otra parte, la biodiversidad es un concepto ecológico jerárquico que comprende los distintos niveles en los que se organiza la vida, desde las variaciones intraespecíficas dentro de poblaciones de una misma especie (diversidad genética), la diversidad entre el número de especies, hasta los tipos de ecosistemas (Harpole, 2010). El estudio de la biodiversidad requiere a su vez el estudio a distintas escalas espaciales (locales y regionales) y temporales para su cuantificación. Basándose en esto, se definen las medidas de la biodiversidad para el estudio de un ecosistema (Zinger *et al.,* 2012) en tres escalas: a) diversidad gamma, a escala del paisaje, considerando el conjunto de comunidades presentes en una región o conjunto de sitios; b) diversidad beta, grado de cambio en la abundancia de las especies entre comunidades; y c) diversidad alfa, riqueza o diversidad de especies dentro de una comunidad, equivalente a una muestra o sitio.

Se puede entender "*comunidad microbiana"* como un ensamblaje de poblaciones de microorganismos que interaccionan entre ellas y con el ambiente, y que co-ocurren en el espacio y el tiempo (Magurran, 2003). La estructura de la comunidad puede ser medida y descrita en términos de composición de especies (diversidad taxonómica), evaluando la riqueza y abundancia relativa de las mismas, las cuales cumplen con funciones especializadas dentro del ecosistema (Atlas y Bartha, 2002). De hecho, los primeros estudios microbiológicos se centraban en la detección, crecimiento, supervivencia y análisis de una función o actividad metabólica concreta de un microorganismo o grupo de microorganismos similares ("gremio") mediante el aislamiento de los mismos desde su hábitat natural en cultivos puros utilizando medios específicos. No es hasta la década de los 60 del siglo XX cuando los microbiólogos empiezan a investigar la importancia de la biodiversidad en la función y estructura de las comunidades microbianas (Hariston *et al.,* 1968).

El interés por la biodiversidad microbiana en sistemas asociados a la industria alimentaria no se desarrollará con amplitud hasta los años 90 del siglo XX, liderados por la Comisión Internacional de Especificaciones de Alimentos (ICMSF). La elaboración de protocolos de detección de riesgos microbiológicos en productos alimenticios y la seguridad de los alimentos fueron los objetivos principales abordados en los primeros estudios de este campo (ICMSF, 1996, 1998a, 1998b y 1998c). En la actualidad, los estudios de biodiversidad en la industria alimentaria también se dirigen a mejorar el rendimiento económico de la producción y la obtención de un producto de calidad vinculándolo al origen (Benito *et al*., 2007; De Angelis *et al*., 2008; Ercolini *et al*., 2008; Gala *et al*., 2008; Gullo y Giudici, 2008; Capozzi *et al*., 2010; Csoma *et al*., 2010; Valmorri *et al*., 2010; Cocolin *et al*., 2011; Cordero-Bueso *et al*., 2011; Tristezza *et al*., 2011). Así, las prospecciones de biodiversidad nos ayudan finalmente a evaluar el potencial económico de un hábitat y las especies que lo pueblan (Chalmers, 1996). El interés de conservar los recursos microbiológicos dependerá de los distintos usos que se le pueda asignar a las especies encontradas o metabolitos producidos por las mismas en procesos biotecnológicos.

En una revisión realizada por Graham H. Fleet en 1999 se describe la información inferida de estudios de ecología microbiana que nos ayudan a alcanzar los objetivos antes mencionados en la industria alimentaria y que pueden ser resumidos en:

- Datos sobre la diversidad y la identificación taxonómica de los microorganismos que colonizan el alimento en cada etapa de su producción.
- Datos de las variaciones cuantitativas de las poblaciones que reflejen cambios durante la producción y la cadena de distribución.
- Información sobre la distribución espacial de los microorganismos en el producto.
- Explicación bioquímica y fisiológica del proceso de colonización.
- Impacto de los factores intrínsecos (propiedades de los alimentos), extrínsecos (factores ambientales), de procesamiento, e implícitos en el crecimiento, supervivencia y actividad bioquímica de los microorganismos (propiedades de los microorganismos).
- Correlaciones entre el crecimiento y actividades metabólicas de los microorganismos individuales y la calidad, higiene y seguridad del producto.

No obstante, la capacidad de dar respuestas confiables a estas premisas ha dependido en gran manera del desarrollo de herramientas adecuadas para el estudio de las comunidades microbianas en su ambiente natural.

3.2 Métodos de estudio de la diversidad microbiana: técnicas moleculares dependientes e independientes de cultivo

En un principio, las técnicas empleadas para la determinación de la diversidad, estructura y dinamismo de las comunidades microbianas se limitaban al estudio de microorganismos cultivables y tecnológicamente relevantes. Estos, tras su aislamiento en medios de cultivo sintéticos más o menos específicos, eran cuantificados en función de los recuentos de unidades formadoras de colonias (UFC) e identificados en base a caracteres fenotípicos, fisiológicos y/o bioquímicos. De hecho, no fue hasta los años 70 del siglo XX cuando Sneath y Sokal, tras la mejora y desarrollo de métodos estadísticos aplicados a la taxonomía numérica, revelaron el escaso poder discriminante de agrupamientos de géneros bacterianos obtenido mediante métodos de clasificación basados en sus rasgos fenotípicos característicos (Sneath y Sokal, 1973).

Por otro lado, el desarrollo de técnicas de biología molecular, basadas principalmente en el análisis de secuencias de ácidos nucleicos (ADN y ARN) permitió establecer relaciones filogenéticas dentro de las bacterias y otros microorganismos, siendo incorporadas en los estudios taxonómicos para la clasificación de microorganismos en base a su genealogía (Woese *et al.,* 1990). Las técnicas moleculares usadas en estudios de diversidad han supuesto un gran avance en el conocimiento de la ecología microbiana, dada la sencillez, rapidez y bajo coste de esta metodología así como la universalidad, sensibilidad, reproducibilidad y poder discriminante de sus resultados en comparación con las pruebas fenotípicas tradicionales. Estas técnicas moleculares incluyen habitualmente la comparación de secuencias genómicas, bien en su totalidad o de determinados genes considerados "marcadores". Estos genes marcadores son aquellos que contienen secuencias muy conservadas y son además virtualmente ubicuos (genes *"housekeeping"*, en inglés). Entre estos genes marcadores destaca el uso de las secuencias del gen *ARNr* 16S, el más difundido, junto con las de otros genes muy conservados tales como *recA*, *rpoA, rpoB*, *pyrH, gyrB, mreB,* etc.

Además, el desarrollo de técnicas de extracción muy eficaces de ácidos nucleicos, no solo desde los microorganismos aislados para su identificación y genotipado ("*DNA-fingerprinting*", en inglés) sino obtenidos directamente desde la matriz alimentaria, y su posterior análisis (técnicas moleculares independientes de cultivo) han permitido tener una visión más realista sobre la diversidad genética asociada a las comunidades microbianas en su

entorno natural. Muestra de todo ello es la identificación de microorganismos "viables no cultivables" (Millet y Lonvaud-Funel, 2000) o aún no cultivados (por ejemplo, especies nuevas de las que aún no conocemos las condiciones para su cultivo) en alimentos.

Hoy en día sabemos que, incluso en matrices alimentarias en las que predominan los microorganismos cultivables, al menos el 25-50% de la comunidad microbiana activa no se puede cultivar *in vitro* (Ampe *et al.,* 1999). Por otro lado, a pesar de los avances logrados gracias al desarrollo de nuevos métodos moleculares independientes del cultivo, estamos lejos de conocer la diversidad microbiana total de una comunidad natural. Esto se debe principalmente a los límites inherentes a los métodos de detección y a la gran cantidad de microorganismos existentes (Curtis *et al.,* 2002; Pommier *et al.,* 2005). Así, se podría decir que ningún método en la actualidad permite responder todas las preguntas, siendo necesario un estudio polifásico en el que se combinen técnicas dependientes e independientes de cultivo si queremos caracterizar la estructura y función de las comunidades microbianas (Juste *et al.,* 2008).

En los siguientes apartados se describen los trabajos científicos y los autores que más recientemente han abordado estudios de diversidad microbiana en aceitunas de mesa mediante técnicas moleculares, agrupándolos según sean dependientes o independientes de cultivo, así como un análisis de los pros y los contras de las distintas estrategias.

3.2.1 Estudio de la diversidad microbiana en fermentaciones de aceitunas de mesa mediante técnicas moleculares dependientes de cultivo

Estos métodos incluyen el aislamiento de microorganismos viables, procedentes tanto de las propias aceitunas como de las salmueras de fermentación, en medios de cultivo y condiciones más o menos selectivos, para su posterior genotipado e identificación mediante técnicas moleculares.

La mayoría de los trabajos presentados hasta la fecha se centran en el estudio de los grupos microbianos más relevantes asociados a la fermentación de aceitunas, a saber: enterobacterias, bacterias lácticas y levaduras. Estos son cuantitativamente monitorizados mediante conteo de UFCs en un medio de cultivo concreto, aunque la selectividad y sensibilidad de estos medios sea relativamente baja. Así, se hace necesario el posterior aislamiento, genotipado e identificación de los microorganismos para alcanzar un poder de discriminación mayor, es decir, a nivel de especie y cepa.

Las técnicas de genotipado más comúnmente empleadas para bacterias inherentes a la fermentación de aceitunas de mesa son las denominadas RAPD-PCR (acrónimo del inglés *"Random Amplification of Polymorphic DNA-PCR"*, es decir, "Amplificación aleatoria de ADN polimórfico") y rep-PCR (del inglés *"Repetitive Element Palindromic-Polymerase Chain Reaction"*, es decir, "Amplificación por reacción en cadena de elementos palindrómicos repetitivos" (Botta y Cocolin, 2012). Por lo general, estos métodos se emplean para agrupar a los aislados microbianos según su homología genética, lo que permite seleccionar luego entre ellos cepas representativas para su posterior identificación molecular al nivel de género y especie.

La identificación molecular de bacterias implica habitualmente la obtención y el análisis de la secuencia del gen *ADNr* 16S. En los casos de especies filogenéticamente cercanas e indistinguibles según la homología de sus secuencias para este gen, el análisis se complementa, si es posible, con análisis por PCR de genes específicos. Así, por ejemplo, la PCR múltiple de amplificación del gen *recA* desarrollada por Torriani *et al*. (2001) es muy utilizada en este área (Botta y Cocolin, 2012) puesto que permite discriminar entre las especies *L. pentosus, Lactobacillus plantarum* y *Lactobacillus paraplantarum,* las cuales son las especies bacterianas más representativas de la ecología bacteriana de la fermentación de aceitunas de mesa.

Por otro lado, la técnica molecular de identificación de levaduras más utilizada en los estudios llevados a cabo sobre aceitunas de mesa es la PCR combinada con RFLP (del inglés *"Restriction Fragment Length Polymorphism"*, es decir, Polimorfismos de Longitud de Fragmentos de Restricción), que implica la amplificación por PCR de las regiones ITS-5.8S ribosomales y el posterior estudio de los tamaños de los fragmentos de restricción obtenidos con determinadas enzimas (Esteve-Zarzoso *et al*., 1999; Botta y Cocolin, 2012). La comparación entre los perfiles de restricción obtenidos a partir de los distintos aislados permite la agrupación de los mismos y su diferenciación a nivel de especie. Si el grado de discriminación obtenido no es suficiente con esta técnica, se recurre a la secuenciación de los dominios D_1/D_2 del gen ribosomal 26S y posterior comparación de la secuencia obtenida en los bancos de datos oportunos (Hurtado *et al.,* 2008; Nisiotou *et al.,* 2010a; Muccilli *et al.,* 2011).

Más recientemente, como alternativa a las descritas más arriba, se ha utilizado la técnica PFGE (*"Pulsed-Field Gel Electrophoresis"*, es decir, Electroforesis en Gel de Campo Pulsado). Esta técnica ha sido aplicada, por ejemplo, para la identificación tanto de bacterias como de levaduras aisladas de la superficie de aceitunas negras al estilo griego por Doulgeraki *et al*., (2012).

En resumen, los fundamentos de las técnicas de genotipado mencionadas más arriba son los siguientes:

- **RAPD-PCR** (Williams *et al.,* 1990): mediante esta técnica, basada en la PCR, se reconocen y amplifican secuencias al azar dentro del ADN genómico que posteriormente se separan mediante electroforesis en geles de agarosa o acrilamida. Se obtiene así una "huella genética" o perfil de bandas característico de los microorganismos aislados. Su mayor ventaja es que, al tratarse de una amplificación arbitraria de fragmentos de DNA, no es necesario conocer previamente el genoma de los microorganismos analizados. Para la amplificación por PCR incluida en esta técnica se requieren unas condiciones poco restrictivas (baja temperatura de anillamiento), puesto que se utilizan cebadores de secuencia aleatoria y de longitud corta (alrededor de 10 pb).

- **rep-PCR** (Versalovic *et al*., 1991): utiliza la PCR con cebadores (35-40 pb) específicos de las regiones de secuencias repetitivas (regiones "rep") descritas en los genomas microbianos. Es una técnica de obtención de una "huella genética" similar a la RAPD-PCR pero en la que se utilizan unas condiciones de anillamiento en la PCR más restrictivas y un único cebador de secuencia específica.

- **RFLP-PCR** (Huffman *et al*., 1992): técnica altamente reproducible que consiste en la comparación de patrones de restricción de diferentes regiones de la zona ribosomal. Para obtener una gran cantidad de copias de la región de interés, la zona seleccionada se amplifica mediante PCR y, posteriormente, el fragmento amplificado se digiere con diferentes endonucleasas de restricción produciendo así un patrón de bandas específico para cada especie.

- **PFGE** (Schwartz y Cantor, 1984): sistema de genotipado que se basa en el análisis del ADN cromosómico completo. Esta técnica tiene como objetivo la fragmentación del genoma microbiano mediante enzimas de restricción muy específicas y que cortan en

pocos puntos el genoma, obteniéndose así fragmentos de gran tamaño. La técnica se apoya en la utilización de un dispositivo diseñado por Schwartz y Cantor (1984) para separar estos grandes fragmentos de restricción mediante electroforesis en geles de agarosa. En este dispositivo, el campo eléctrico cambia periódicamente de dirección, lo que facilita la migración de grandes fragmentos de ADN y su separación por tamaño a través del gel. Los patrones de bandas obtenidos, denominados PFPs (*"Pulsed-Field Profiles"*, es decir, perfiles de campo pulsado), son característicos de cada aislado. Este método presenta un alto poder discriminante a nivel intraespecífico, además de una buena reproducibilidad respecto al obtenido con RAPD-PCR y rep-PCR (Vandamme *et al.,* 1996). Sin embargo, es laborioso y caro, por lo que su aplicación no resulta rentable cuando se trata de analizar un elevado número de aislados (Blaiotta *et al*., 2001).

La principal ventaja que suponen estas estrategias de genotipado para la microbiología de alimentos es que permiten hacer estudios que correlacionan el crecimiento, supervivencia y actividad metabólica de organismos individuales con la calidad, higiene y seguridad del producto. En este sentido, múltiples estudios se han realizado para la selección de cepas con características deseables para su empleo como cultivos iniciadores y, posteriormente, la realización de ensayos para monitorizar la capacidad real de colonización del alimento del cultivo iniciador y estudiar su efecto en el producto final (Corsetti *et al*., 2012; Heperkan, 2013).

Por otro lado, las desventajas que presenta esta metodología están relacionadas con la denominada "gran anomalía del cultivo en placa" (Goodman *et al.,* 1998), esto es, la diferencia observada entre el número de células viables presentes en una muestra y el número de colonias recuperadas en medio de cultivo (Felske *et al.,* 1999; Eilers *et al.,* 2000; Buée *et al.,* 2009). También es de destacar la dificultad de recuperar la diversidad total de una comunidad microbiana debido a los problemas para obtener cultivos puros de las distintas especies de la comunidad y la limitada capacidad de reproducir las condiciones del entorno natural del que fueron aisladas. Además, esta metodología es muy laboriosa y costosa tanto en tiempo como económicamente. Aun así, el esfuerzo continuo a lo largo de los años ha aumentado la probabilidad de aislamiento de la mayor diversidad posible mediante la combinación de medios de cultivo, la adición a los mismos de nutrientes no convencionales o sustancias inhibitorias, y la aplicación de largos periodos de incubación. El desarrollo de

programas informáticos para el almacenamiento y análisis de distintos tipos de datos biológicos (caracteres fenotípicos, secuencias de ADN, perfiles electroforéticos,..), como por ejemplo ofrece el programa Bionumerics (http://www.applied-maths.com/bionumerics), así como el abaratamiento y mejora de las técnicas de secuenciación de ADN y la disponibilidad de bancos de datos actualizados, mejor implementados y accesibles a través de Internet (EMBL, GenBank, etc.), han agilizado y mejorado mucho la utilización de estas metodologías en estudios de diversidad microbiana.

3.2.2 *Estudio de la diversidad microbiana en fermentaciones de aceitunas de mesa mediante técnicas moleculares independientes de cultivo*

Las técnicas moleculares independientes de cultivo ofrecen la posibilidad de estudiar los microorganismos en un ecosistema específico sin la necesidad de su cultivo previo. Su aplicación en el campo de la microbiología de alimentos empezó a finales de los años 90 del siglo XX, orientados a la monitorización de la microbiología de alimentos fermentados y la detección de patógenos (Cocolin *et al*., 2013a), si bien el primer estudio en aceitunas de mesa fue publicado por Abriouel *et al*. (2011). En este primer estudio se monitorizaba la diversidad y dinamismo de bacterias, arqueas y eucariotas (levaduras y mohos) en las salmueras de fermentación de aceitunas de la variedad Aloreña elaboradas al natural, es decir, sin tratamiento alcalino previo, mediante electroforesis en gel con gradiente desnaturalizante (DGGE, *"Denaturing Gradient Gel Electrophoresis"*, Fischer y Lerman, 1983). Este estudio permitió la detección de microorganismos nunca antes aislados de fermentaciones de aceitunas de mesa, tales como *Thalassomonas agarivorans*, una bacteria marina probablemente procedente de la sal utilizada para elaborar la salmuera.

Estudios posteriores sobre la diversidad microbiana en aceitunas de mesa mediante PCR-DGGE fueron realizados por Muccilli *et al*. (2011), centrado en el estudio de levaduras en aceitunas verdes sicilianas, así como por Randazzo *et al.* (2012), Cocolin *et al.* (2013b) y Tofalo *et al.* (2014), dirigidos al estudio de la huella genética de la comunidad bacteriana a lo largo de la fermentación de aceitunas verdes de mesa de distintos cultivares italianos.

Otras estrategias moleculares independientes de cultivo que han sido utilizadas en el estudio de la microbiota de aceitunas de mesa son FISH (*"Fluorescence In Situ Hybridization"*, es decir, hibridación fluorescente *in situ*), aplicada por Ercolini *et al.* (2006) para la localización espacial de células de *L. plantarum* en la superficie de las aceitunas,

RFLP-PCR, utilizada por Aponte *et al.* (2010), estudio que permitió detectar una mayor diversidad de especies respecto a las técnicas dependientes de cultivo en aceitunas verdes sicilianas, y la pirosecuenciación, con la que Cocolin *et al.* (2013b) evaluaron los cambios en la estructura de la comunidad bacteriana a lo largo de la fermentación de aceitunas verdes de mesa y que, a pesar de tener un límite teórico de detección mayor incluso que la PCR-DGGE, presentó una capacidad muy similar de discriminación.

Como puede observarse por lo expuesto más arriba, pese a la amplia diversidad de técnicas independientes de cultivo descritas, son relativamente pocos los trabajos publicados que las aplican a la microbiología de la fermentación de aceitunas de mesa, siendo la PCR-DGGE la metodología más frecuentemente utilizada para ello. Basada en el análisis de la heterogeneidad de las secuencias de ácidos nucleicos (ADN y ARN), la PCR-DGGE es también el método más utilizado para analizar fragmentos de ADN amplificados por PCR procedentes de muestras ambientales para comparar la diversidad de comunidades microbianas en el área de la microbiología de alimentos (Cocolin *et al.*, 2013a). Más en detalle, esta técnica consta de los siguientes pasos: i) extracción del ADN/ARN total de la comunidad microbiana presente en la muestra; ii) amplificación por PCR de secuencias de 200-700 pb; iii) separación electroforética de los amplicones resultantes mediante electroforesis en gel de poliacrilamida con un gradiente desnaturalizante químico (de urea y formamida) creciente y en paralelo al campo migratorio, a temperatura constante (Muyzer *et al*., 1993; Muyzer y Smalla, 1998); y iv) procesamiento y análisis de la huella digital obtenida. Los cebadores utilizados en la PCR correspondiente pueden ser bien cebadores universales de regiones variables que permiten una amplia resolución taxonómica (ej. Bacteria, Archaea o Eucarya), o cebadores específicos de grupo (funcional) para detectar y/o cuantificar sólo microorganismos específicos o genes de interés (Juste *et al*., 2008). Los fragmentos amplificados mediante PCR poseen secuencias de la misma longitud aunque específicas, siendo separados según su temperatura de fusión (Tm, "*melting temperature*") en el gel. El comportamiento de desnaturalización del fragmento dependerá, por tanto, del contenido G+C y la secuencia nucleotídica del mismo. Así, los fragmentos amplificados de ADN conservan su estructura de doble hélice hasta que alcanzan las condiciones de desnaturalización del dominio de menor temperatura de fusión en el que se inicia la desnaturalización y apertura de la cadena, reduciéndose su velocidad de migración en el gel. La adición al extremo 5' del cebador F (*"forward"*, cebador directo) de una secuencia rica en

G+C (40–45 bases) evita que las hebras de ADN se separen totalmente (Sheffield *et al.*, 1989).

Los gradientes de desnaturalización deben de ser adaptados a la muestra de estudio para conseguir una óptima separación de los fragmentos (Ogier *et al*., 2002). La posición relativa de cada banda en el gel, correspondiente a un amplicón determinado, se correspondería con una especie microbiana concreta. Además, esta técnica permite teóricamente incluso hacer análisis de mutaciones, es decir, apreciar diferencias de migración entre secuencias que difieren entre sí en un único nucleótido. Otras ventajas que ofrece esta técnica para su aplicación en estudios de diversidad son la capacidad para establecer comparaciones entre comunidades microbianas en el espacio, monitorizar la dinámica poblacional en el tiempo (Sun *et al*., 2004; Camu *et al*., 2007), y detectar microorganismos no cultivables. Además, esta metodología, frecuentemente utilizada para amplificar regiones variables del gen *ADNr* 16S mediante el uso de cebadores tanto universales como específicos (Randazzo et al., 2002; Ercolini *et al*., 2003), puede utilizarse para seguir productos derivados de secuencias de ARNm obtenidos mediante retrotranscripción, permitiendo así el seguimiento específico de las poblaciones microbianas metabólicamente activas en cada muestra (Otawa *et al*., 2006; Dar *et al*., 2007). De hecho, Cocolin *et al.,* (2013b) apreciaron diferentes agrupamientos de las huellas microbianas obtenidas en base a la amplificación a partir de extracciones de ADN o ARN de las muestras, permitiéndoles así distinguir entre poblaciones microbianas viables y no viables.

El análisis numérico de los patrones de bandas tras la digitalización y procesamiento de las imágenes obtenidas a partir de la PCR-DGGE suele incluir estudios de diversidad y el cálculo de los coeficientes de similaridad entre los perfiles obtenidos. Asimismo se incluyen la identificación y monitorización de las bandas obtenidas en base a su posición relativa en el gel. Así, la identificación de las bandas de los perfiles electroforéticos pueden ser obtenidas mediante el análisis de las posiciones relativas de dichas bandas, comparándolas con las posiciones de las bandas de patrones de especies microbianas de referencia. Cuando esto no es posible, o existen dudas, se puede recurrir a la extracción, purificación, reamplificación y secuenciación de las bandas de interés. La homología de la secuencia de ADN obtenida con las depositadas en los bancos de datos relevantes puede indicarnos la especie microbiana problema.

Por otra parte, la información conseguida de esta manera no siempre es suficiente para alcanzar una clasificación taxonómica precisa (Øvreås, 2000). Además, se pueden dar casos de comigración de fragmentos con secuencias diferentes pero con igual movilidad electroforética (Sekiguchi *et al.,* 2001). También, aunque el análisis estadístico de los perfiles electroforéticos obtenidos tras la digitalización de las imágenes del gel es relativamente sencillo, pueden existir problemas de reproducibilidad (Powell *et al*., 2005) o de baja sensibilidad que lo dificulten. En el último caso, no sería posible detectar las poblaciones microbianas menos abundantes. Otras desventajas de esta técnica se derivan de los posibles sesgos en el procedimiento de extracción de ácidos nucleicos de la muestra, dado que no todos los microorganismos presentan, por ejemplo, el mismo comportamiento frente a las condiciones de lisis inherentes a la extracción de ADN/ARN total de la muestra, o presentan una respuesta muy distinta a la propia amplificación por PCR. También hay que tener en consideración el número de copias y la posible micro-heterogeneidad en la secuencia del gen marcador que se utilice para la identificación, de tal forma que la huella microbiana obtenida al final pudiera no ser del todo representativa de la microbiota presente en la muestra.

3.3 Taxonomía y concepto de especie microbiana

La sistemática microbiana es el estudio científico de la diversidad y clases de los microorganismos y sus relaciones mutuas (Goodfellow y O'Donnell, 1993). Esta disciplina comprende tres actividades interrelacionadas entre sí: la clasificación, la nomenclatura y la identificación.

La clasificación es la organización de organismos en grupos taxonómicos en base a la semejanza de sus caracteres fundamentales o su parentesco evolutivo. La nomenclatura es el proceso de asignar nombre a los grupos taxonómicos y se realiza siguiendo las reglas del Código Internacional de Nomenclatura Bacteriológica (Lapage *et al*., 1992) para las bacterias mientras que la nomenclatura de las levaduras está supeditada al Código Internacional de Nomenclatura para algas, hongos y plantas, recientemente modificado tras la Declaración de Ámsterdam sobre Nomenclatura de Hongos (Hawksworth *et al*., 2011). Finalmente, la identificación determina si, tras la caracterización de un aislado, éste pertenece a un taxón preexistente o si debe ser clasificado como un nuevo taxón. Por consiguiente, la clasificación precede a la identificación y ambas dependen de los avances tecnológicos.

Los rangos taxonómicos están organizados de forma jerárquica en las siguientes categorías: reino, clase, orden, familia, género y especie. Es esta última, la especie, la unidad taxonómica básica y más importante en la sistemática microbiana, mientras que los demás rangos superiores son abstractos y artificiales (Hull, 1997).

3.3.1 Taxonomía bacteriana

En la actualidad no existe un sistema oficial y único para la clasificación bacteriana, si bien la denominada taxonomía polifásica es la más ampliamente aceptada por la comunidad científica (Brenner *et al*., 2001). Ésta consiste en integrar los resultados de caracteres independientes, tanto fenotípicos como genotípicos y filogenéticos, para garantizar la identificación completa de un microorganismo (Colwell, 1970; Vandamme *et al*., 1996). Así, la especie bacteriana se define como "un grupo monofilético y genómicamente coherente de organismos individuales que muestran un elevado grado de similitud global con respecto a muchas características independientes, y que es diagnosticable por unas propiedades fenotípicas discriminativas" (Rosselló-Móra y Amann, 2001). Este concepto filofenético de especie queda, por tanto, restringido al conjunto de microorganismos cultivables en cultivo puro (Vandamme *et al*., 1996).

Otra peculiaridad de la sistemática bacteriana es que cada especie está vinculada a las características de una cepa designada "tipo". Esta cepa se utiliza como referencia para la caracterización taxonómica, siendo necesario que se conserve de forma viable y que esté disponible para la comunidad científica, para lo cual debe ser depositada en al menos dos colecciones de microorganismos diferentes ubicadas en países distintos (Tindall *et al*., 2010). Por otra parte, es aconsejable que la identificación de una nueva especie no esté basada en un único espécimen (cepa) (Christensen *et al*., 2001). En general, la descripción de una nueva especie incluye alguna información sobre su hábitat natural (localización y características fisicoquímicas de la muestra de la que fueron aisladas), así como su caracterización fenotípica y genotípica siguiendo una metodología más o menos establecida y que se detalla a continuación.

3.3.1.1 Caracterización fenotípica

La caracterización fenotípica refleja la expresión observable de la información contenida en el genoma microbiano, así como el fruto de procesos epigenéticos (Zhi *et al.,* 2012). Habitualmente incluye la descripción de las características de las colonias (forma, tamaño,

color, indicios de motilidad o *"swarming"*) y de las propias células. En este último caso, se incluyen tanto características morfológicas (forma, tamaño, movilidad, agrupamiento, presencia de esporas o cuerpos de inclusión, etc.), como de respuesta a tinciones específicas (Gram, esporas, cápsulas, etc.). Además, se realizan pruebas fisiológicas, encaminadas a conocer las condiciones óptimas de crecimiento y la tolerancia a distintos factores de estrés (pH, temperatura, relación con el oxígeno, requerimientos iónicos específicos, resistencia a antibióticos, etc.), así como pruebas bioquímicas, dirigidas a conocer el metabolismo específico de la bacteria incluyendo la actividad de determinadas enzimas o la utilización de distintas fuentes de carbono o nitrógeno. Para esto último se suelen utilizar actualmente kits comerciales miniaturizados como API (Biomérieux) o BIOLOG (Biolog Inc.). También se pueden incluir pruebas quimiotaxonómicas, para determinar la composición de la pared, la membrana o el citoplasma celular.

3.3.1.2 Caracterización genotípica

La caracterización genotípica implica el estudio de la información contenida en el material genético de la célula, lo que requiere el empleo de distintas metodologías de análisis del ADN. Estas metodologías incluyen habitualmente el análisis de la secuencia del gen que codifica la subunidad pequeña del ribosoma (*ADNr* 16S), la hibridación del ADN total con cepas bacterianas de referencia, la determinación del contenido en G+C del ADN genómico e incluso, más recientemente, la secuenciación del genoma completo (Tindall *et al.,* 2010).

- Análisis de la secuencia del gen ribosomal *ADNr* 16S

La demostración de que los miembros de una nueva especie pertenecen a un linaje monofilético se realiza mediante inferencia filogenética basada principalmente en el análisis comparativo de la secuencia completa del gen ribosomal *ADNr* 16S (Ludwig y Klenk, 2001).

Las bondades del gen *ADNr* 16S como marcador molecular en estudios de filogenia entre procariotas se debe a las siguientes características:

– Gen altamente conservado, funcionalmente constante y ubicuo.

– No está implicado en fenómenos de transferencia horizontal de genes, por lo que se consideran estables.

- Existe una relación empírica no-lineal entre la similitud que presentan los genomas completos y el porcentaje de identidad de las secuencias del gen *ADNr* 16S, por lo que éste refleja la distancia evolutiva entre los microorganismos.

- Tamaño adecuado de la secuencia, es decir, ni demasiado pequeño, que no aportaría suficiente información genética (como le ocurre a la subunidad 5S de los genes ribosomales), ni demasiado grande, que impondría ciertas dificultades a su secuenciación (caso de la subunidad ribosomal 23S).

- Alto grado de resolución taxonómico. Así, dos aislados bacterianos con semejanzas de secuencia inferiores a 98,7% presentan una relación a nivel genético baja y se pueden considerar especies distintas (Stackebrandt y Ebers, 2006). En cambio, para afirmar que dos aislados bacterianos con niveles de semejanza superiores al 98,7% pertenecen a especies distintas debe estar avalado por los resultados de hibridación de su ADN total y su caracterización fenotípica.

Sin embargo, es a veces difícil llegar a una identificación a nivel de especie o subespecie mediante el análisis exclusivo de la secuencia del *ADNr* 16S (Stackebrandt y Ebers, 2006). Entre las recomendaciones actuales para conseguir una apropiada reconstrucción genealógica basada en la secuencia del gen *ADNr* 16S (Rosselló-Móra y Amann, 2015) está en primer lugar comparar (alinear mediante un algoritmo tal como ClustalW, por ejemplo) las secuencias obtenidas, que han de ser completas y de buena calidad, con aquellas secuencias depositadas en los bancos de datos de especies conocidas. En esta comparación debemos incluir las secuencias pertenecientes a las cepas tipo, las cuales se pueden obtener para cada especie, por ejemplo, del servidor EzTaxon (accesible en http://www.eztaxon.org) (Chun *et al.,* 2007). La estructura secundaria de la molécula de ARNr 16S es también un factor a tener en cuenta en estas comparaciones. Finalmente, para la construcción de los árboles filogenéticos se aconseja utilizar múltiples algoritmos, siendo el Método de unión de vecinos (NJ, *"Neighbor Joining"*), Máxima Parsimonia (MP, "*Maximum parsimony*") y Máxima Verosimilitud (ML, "*Maximum Likelihood*") los más habituales. Además, es conveniente aplicar distintos filtros o conjuntos de secuencias y finalmente evaluar la robustez del árbol resultante mediante ensayos de replicado ("*bootstrapping*").

- Hibridación de genomas (ADN:ADN)

Aún hoy, es considerada el estándar de referencia en taxonomía para delimitar especies. Consiste en calcular la similitud de nucleótidos entre genomas completos en base a la capacidad de re-asociación del ADN en condiciones experimentales adecuadas de hibridación (Stackebrandt y Goebel, 1994). En este caso, el parámetro utilizado para medir el grado de reasociación ADN: ADN es la diferencia en el punto medio de las curvas de desnaturalización térmica del ADN homodúplex y heterodúplex. Se ha establecido empíricamente que, bajo unas condiciones de hibridación óptimas, cepas de una misma especie presentan una diferencia en su temperatura media de desnaturalización inferior a 5ºC, lo que se corresponde con una tasa relativa de unión del 70% entre las secuencias (Wayne *et al.,* 1987; Ward, 1998). Aunque la metodología original para hacer estos cálculos es bastante engorrosa y, a veces, poco fiable, González y Saiz-Jiménez (2005) han desarrollado un método fluorimétrico usando PCR cuantitativa a tiempo real que es más asequible.

- Contenido G+C del ADN genómico

El contenido en G+C del ADN genómico de una bacteria, expresado como porcentaje, es una medida de la composición de bases de dicho genoma. Se considera una característica necesaria para la descripción de un género nuevo. Empíricamente, se ha demostrado que microorganismos que difieren en más de un 10% en su contenido G+C, no deberían ser considerados como miembros del mismo género, mientras que si difieren en más de un 5% pertenecen a especies distintas (Vandamme *et al*., 1996). Sin embargo, un porcentaje similar no implica necesariamente que dos cepas estén estrechamente relacionadas entre sí, dado que no se tiene en cuenta la secuencia lineal de bases del ADN genómico. De nuevo, un método fluorimétrico asociado a PCR cuantitativa a tiempo real desarrollado por González y Saiz-Jiménez (2002) facilitan estos cálculos.

- Secuenciación del genoma completo

Los recientes avances tecnológicos dentro de las técnicas de secuenciación están haciendo posible la comparación de genomas completos entre microorganismos. En este sentido se ha comprobado, por ejemplo, que la media de identidad nucleotídica (ANI, "*Average Nucleotide Identity*") entre micoroorganismos de una misma especie ha de ser mayor del 94%. También se ha propuesto la utilización del genoma completo como material tipo para la descripción de procariotas (Rosselló-Móra y Amann, 2015), con lo

que se podría incluir la descripción de microorganismos no cultivables o los endosimbiontes, que actualmente son nombrados con el estatus provisional de "*Candidatus*" (Murray y Scheifer, 1994; Murray y Stackebrandt, 1995).

3.3.2 Taxonomía de levaduras

Las levaduras se definen como hongos de morfología predominantemente unicelular que se reproducen por gemación o fisión y cuyo estado sexual, de conocerse, no se halla encerrado dentro de un cuerpo frutífero (Kurtzman *et al*., 2011a). Estos microorganismos no conformaban por si mismos una entidad taxonómica propiamente dicha y su estudio, como el del resto de hongos, se basaba tradicionalmente en pruebas morfológicas, fisiológicas, y bioquímicas. Sin embargo, este estado de cosas cambió significativamente desde la proposición de Taylor *et al.* (2000) de integrar el concepto de especie filogenética con concordancia genealógica como requisito para delimitar el reconocimiento de especies en hongos a partir del análisis de secuencias de múltiples *loci*-independientes y, paralelamente, desde la adopción del concepto "código de barras de ADN" (*"DNA barcode"*, Hebert *et al*., 2003) para la rápida identificación de los hongos (Schoch *et al*., 2012).

En la actualidad, el marcador del "código de barras de ADN" para los hongos es la región entre los espacios transcritos internos (ITSs, *"Internal Transcribed Spaces"*) de los genes ribosomales. Se calcula que, mediante este criterio, existe una probabilidad del 73% de obtener una correcta identificación a nivel de especie, teniendo, por tanto, una resolución similar a los marcadores establecidos para la discriminación de especies en plantas (Schoch *et al*., 2012). Por otra parte, el desarrollo nuevas técnicas moleculares así como la secuenciación de genomas han permitido esclarecer las relaciones filogenéticas existentes entre las levaduras. A este respecto, y resumiendo a grandes rasgos los resultados obtenidos tras la reciente reclasificación de las especies y géneros conocidos, se puede concluir que todas las especies de ascomicetes con fase levaduriforme se agrupan en un único clado monofilético, denominado *Saccharomycotina* (Kurtzman y Robnett, 2013), mientras que las especies de basidiomicetes con fase levaduriforme se encuentran diseminadas entre los tres principales linajes descritos para estos hongos, a saber *Pucciniomycotina, Ustilaginomycotina* y *Agaricomycotina* (James *et al*., 2006; Hibbett *et al*., 2007; Boekhout *et al*., 2011).

El conocimiento de las relaciones filogenéticas entre las especies también ha impulsado la reciente modernización en las reglas de nomenclatura implementadas en el Código

Internacional de Nomenclatura para algas, hongos y plantas. Siendo las principales medidas adoptadas las siguientes (Hibbett y Taylor, 2013):

- El abandono definitivo del sistema dual de nomenclatura latinizado basado en la clasificación de los hongos según los caracteres derivados de las estructuras reproductivas (Alexopoulos *et al*., 1996), el cual permitía crear un nombre para la forma asexuada (género anamórfico) y otro para la sexual (género teleomórfico) de una misma especie.

- La no prioridad en la circunscripción de género del nombre teleomórfico sobre el anamórfico. Una implicación adicional a este respecto está siendo el desarrollo de listas de nombres para obtener el estatus de "nombre genérico protegido" que deberán ser ratificados en el próximo congreso de botánica internacional (IBCXIX) que se celebrará en China en el año 2017 (Hawksworth *et al*., 2012).

- Se posibilita la descripción de especies nuevas en publicaciones no impresas y se incorpora la obligación del registro de los nombres nuevos en bases de datos de acceso público tales como Index Fungorum o Mycobank.

- La no restricción en la forma del espécimen tipo físico a depositar, no siendo necesario que éste sea representativo o esté completo. Este punto fue propuesto por Kirk *et al.* (2012) tras la descripción de la especie *Piromyces cryptodigmaticus,* lo cual posibilita el futuro desarrollo de una taxonomía puramente basada en estudio de secuencias.

Finalmente, el Código Internacional de Nomenclatura para algas, hongos y plantas, a diferencia del Código Internacional de Nomenclatura Bacteriológica, no especifica el procedimiento técnico para el reconocimiento de especies nuevas (Hibbett y Taylor, 2013), si bien se suele seguir también el método polifásico que contempla una caracterización fenotípica y genotípica. No obstante, en la delimitación de especie nueva y a la hora de la circunscripción del género al que pertenece, tienen prioridad los resultados que se obtienen de la caracterización genotípica.

3.3.2.1 Caracterización fenotípica

La caracterización fenotípica de especies nuevas de levaduras se fundamenta en la presencia de una fase de crecimiento unicelular, si bien la caracterización morfológica

también incluye una descripción del crecimiento de las colonias en cajas de agar, la morfología de las células asexuales y el tipo de reproducción sexual. Esta caracterización se completa con pruebas fisiológicas y bioquímicas, principalmente: fermentación de carbohidratos, utilización de distintas fuentes de carbono y nitrógeno, requerimiento de vitaminas, tolerancia a altas temperaturas (fijar la temperatura máxima de crecimiento), al ácido acético y osmotolerancia (capacidad de crecer a altas concentraciones de sal y azúcar), así como actividades enzimáticas, tales como hidrólisis de la urea, actividad lipasa y capacidad de licuefacción de la gelatina, entre otras (Yarrow, 1998; Kurtzman *et al*., 2011b).

3.3.2.2 Caracterización genotípica

La caracterización genotípica rutinaria para la identificación de especies nuevas de levaduras consiste en determinar la divergencia en las secuencias de distintas zonas genómicas, a saber:

- La región de los dominios D_1/D_2 del gen ribosomal de la subunidad mayor 26S (*ADNr* 26S). Con una longitud aproximada de 450-600 nucleótidos, esta región está flanqueada por secuencias altamente conservadas que pueden ser amplificadas mediante una misma pareja de cebadores para todas las especies (Kurtzman y Robnett, 1998; Fell *et al*., 2000). Sin embargo, este marcador es menos discriminativo que el ITS para algunos linajes de hongos sin fase levaduriforme conocida, por lo que no se utiliza como "código de barras" de los hongos en general (Schoch *et al*., 2012).

- La región de los ITSs de los genes ribosomales. Presenta un tamaño similar al de la región D_1/D_2 del gen *ADNr* 26S. Esta región separa el gen ribosomal de la subunidad menor 18S del de la subunidad mayor 26S y, a su vez, está dividida por el gen *ADNr* 5.8S, el cual es altamente conservado y no debería ser incluido cuando se analiza la divergencia entre las secuencias de los ITSs.

La aptitud demostrada de estas regiones en los estudios genealógicos de levaduras se debe a que sus secuencias exhiben grandes diferencias interespecíficas y un bajo polimorfismo intraespecífico. Ambos tipos de secuencias muestran similar resolución en la identificación de especies, con ligeras variaciones taxón-específicas, por lo que se recomienda el uso de ambos tipos de secuencias para la descripción de una especie nueva. De forma general, se puede decir que cepas de una misma especie presentan una divergencia en las secuencias D_1/D_2 e ITS no superiores al 1% (Kurtzman y Robnett, 1998; Fell *et al*., 2000; Scorzetti *et al*., 2002;

Sugita *et al*., 2002). Sin embargo, existen algunas excepciones a este punto de corte que pueden ser debidas a hibridaciones interespecíficas, diferente tasa de sustitución de nucleótidos u otros cambios genéticos (Kurtzman *et al*., 2015).

3.3.3 Potencial de la industria de aderezo de aceitunas de mesa para el aislamiento e identificación de nuevas especies microbianas

El desarrollo de técnicas moleculares basadas en el genotipado y la secuenciación de zonas específicas del genoma, así como la elaboración de bases de datos para el análisis molecular de microorganismos, han facilitado la identificación de nuevas especies microbianas considerablemente (Sutcliffe *et al*., 2012; Kurtzman *et al*., 2015). El aumento en el número de hallazgos de nuevas especies está también relacionado con la actual concienciación por parte de sectores económicos acerca de la importancia de los recursos genéticos ligados a la diversidad biológica (Bartkowski *et al*., 2015), lo que ha dado lugar a la bioprospección de ecosistemas muy diversos. Esta bioprospección incluye ecosistemas fermentativos de interés dentro de la industria agroalimentaria, como es el caso de la industria de aderezo de aceitunas de mesa. En este sentido, en los últimos quince años ha aumentado significativamente el número de nuevas especies, tanto de levaduras como de bacterias, originalmente aisladas durante la fermentación de diversas elaboraciones de aceitunas de mesa o halladas en efluentes de estas industrias.

Nuevas especies de levaduras aisladas de fermentaciones de aceitunas de mesa han sido *Saccharomycopsis olivae* (Jacques *et al*., 2014), *Citeromyces nyonsensis* (Casaregola *et al*., 2013) y *Candida olivae* (Nisiotou *et al*., 2010b). Estas especies fueron aisladas de salmueras de fermentación de aceitunas negras al natural (estilo Griego) en las que, a diferencia de las aceitunas verdes al estilo Español, las levaduras constituyen la microbiota responsable de la fermentación (Nychas *et al*., 2002). A pesar de que ninguna de estas especies de levaduras pudo ser considerada dominante a lo largo de la fermentación (Coton *et al*., 2006; Nisiotou *et al*., 2010a), estos resultados avalan el hecho de que las fermentaciones de aceitunas de mesa siguen siendo un hábitat muy poco estudiado en cuanto a la diversidad de levaduras (Arroyo-López *et al*., 2008).

Por otra parte, las nuevas especies bacterianas *Bacillus sp.* WW3-SN6 (Ntougias y Russell, 2000), *Alkalibacterium olivoapovliticus* (Ntougias y Russell, 2001) y *Virgibacillus olivae* (Quesada *et al.,* 2007) fueron aisladas de efluentes (fundamentalmente aguas de

lavado) procedentes de la elaboración de aceitunas de mesa elaboradas al estilo Español. Estas bacterias resultaron ser Gram-positivas, halotolerantes y, a excepción de la especie *V. olivae,* capaces de desarrollarse a valores de pH alcalino extremos.

CAPÍTULO 4
PUBLICACIONES

4.1 Microbial diversity and dynamics of Spanish-style green table-olive fermentations in large manufacturing companies through culture-dependent techniques.

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Microbial diversity and dynamics of Spanish-style green table-olive fermentations in large manufacturing companies through culture-dependent techniques

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ABSTRACT

We have studied the microbiota associated to Spanish-style green olive fermentations, attending to its dynamics along the time. Twenty 10-tonne fermenters were selected from two large table-olive manufacturing companies in southern Spain. While culture-dependent methodology was used to isolate the microorganisms, molecular methods were used to identify the isolates. A total of 1070 isolates were obtained, resulting in 929 bacterial and 141 yeast isolates. Thirty seven different bacterial species were isolated, belonging to 18 different genera, while 12 yeast species were isolated, belonging to 7 distinct genera. This fermentation was dominated by the species *Lactobacillus pentosus*, while its accessory microbiota was variable and depended on the fermentation stage and the actual fermentation yard ("*patio*"). It was noticeable the abundance of lactic acid bacteria isolates, belonging to 16 different species. Twenty bacterial species were isolated for the first time from Spanish-style green olive fermentations, while 17 had not been described before in any table olive preparation. The genera *Brachybacterium*, *Paenibacillus*, *Sporolactobacillus*, *Paracoccus* and *Yersinia* had not been cited before from any table olive preparation. *Saccharomyces cerevisiae* and *Candida thaimueangensis* appeared to dominate the yeast microbiota. *Candida butyri/asseri* and *Rhodotorula mucilaginosa* had not been described before from Spanish-style preparations, while *Saturnispora mendoncae* was isolated for the first time from any table olive preparation. Biodiversity was analysed through different alpha and beta indexes which showed the evolution of the microbiota over time.

1. Introduction

Table olives account for the largest volume of fermented vegetables in Western countries, especially in Mediterranean countries (Garrido Fernández et al., 1997). World production reached an average of 2.3 million tons per year in the period 2006–2012 (IOOC, 2012). Although table olives can be prepared for consumption in many different ways, Spanish-style preparation of green olives is one of the three most commercially important worldwide, along with natural black olives and oxidised black olives (Garrido Fernández et al., 1995; Sánchez et al., 2006; Rejano et al., 2010), representing 60% of the world production (Botta and Cocolin, 2012). Spanish-style preparation is characterised by the initial alkali treatment (1.8–3.5% [w/v] NaOH) of the green fruits, which removes bitterness and allows the subsequent growth of lactic acid bacteria (LAB) through the neutralisation and washing of inhibitory phenolic compounds (Rejano et al., 2010). Once removed the alkali, fruits are washed once or twice with water and finally covered with brine (10–12% [w/v] NaCl). In this brine a spontaneous fermentation takes place in which at least three different stages have been identified (Garrido Fernández et al., 1995). During the first stage, usually lasting 3–10 days, fermentation is conducted by the indigenous alkali-tolerant microbiota which contaminates the fruits as well as the environment (De Castro et al., 2002). This microbiota is responsible for lowering the initial high pH (10–11) to values close to 6–7, more appropriate for the growth of LAB, which are also present as contaminants (Sánchez et al., 2001). As soon as LAB take over and grow exponentially, during what it is considered the second stage in this fermentation, pH value drops as a result of their metabolism. Sugars are converted into lactic acid, as the major

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product, as a result of a mainly homolactic fermentation. This is carried out mostly by strains of the species *Lactobacillus pentosus* (De Castro et al., 2002; Rejano et al., 2010; Ruiz-Barba and Jiménez-Díaz, 2012), although in the past this role was attributed to strains of *Lactobacillus plantarum* (Ruiz-Barba et al., 1994; Garrido Fernández et al., 1995; Rejano et al., 2010) as a consequence of previous phenotypic criteria for the classification of species into what it is known as the "*L. plantarum* group", before molecular criteria were applied (Torriani et al., 2001). At the end of the second stage, typically 10–15-day long, pH value is about 4.5 and most sugars have been utilised (Montaño et al., 1993; Garrido Fernández et al., 1995). During the final, third stage of the fermentation all fermentative substrates are exhausted and LAB population declines steadily. Values of pH below 4.0 and free acidity of 0.7–1.2%, mainly as lactic acid, are considered indicative of a good fermentation. These conditions, combined with a NaCl concentration which is at this stage usually raised to 7–8%, should guarantee the long-term preservation of the final product.

Up to date, few comprehensive studies have been carried out on the microbiota of table olive fermentations, especially if we consider modern taxonomic criteria and molecular techniques (Ercolini et al., 2006; Botta and Cocolin, 2012; Cocolin et al., 2013). The aim of this study is to update the knowledge we have about the microbial diversity, in terms of both bacteria and yeast, which is inherent to the Spanish-style fermentation of green olives in large scale table-olive manufacturing companies. For this, we have used culture-dependent techniques for the isolation of the different microorganisms as well as molecular techniques to obtain as precise identifications as possible. We have selected two different large-scale table-olive fermentation yards (known in Spanish as "*patios*"), belonging to two large table-olive manufacturing companies in the province of Seville, southern Spain. In this province, up to 63% of the Spanish national production is concentrated (season 2012/2013; AAO, 2013), so that data obtained should be quite relevant. Actually, this table olive preparation is also known as "Sevillian-style" (Rejano et al., 2010). Finally, our goal is to obtain not only a picture of the microbial diversity along the time of this food fermentation but also get a well characterised collection of microorganisms to be used in the future as a comprehensive bank of wild-type strains for diverse biotechnological uses.

2. Materials and methods

2.1. Origin of the samples and sampling strategy

Samples of Spanish-style green-olive fermenting brines were taken during the 2010–2011 season from two large (4000–8000 tonnes of olives handled per season) table-olive manufacturing companies in the province of Sevilla, south-western Spain. These companies are located *ca.* 35 Km apart from each other. At each company, fermentation was followed in ten fermenters. These were of a total capacity of 10 tonnes of olives and 5500–6000 L of brine, made in polyester and glass fibre. They were all located outdoor, buried in the ground of what it is traditionally called in Spain a "*patio*". The traditional Spanish-style procedure to prepare green olives was followed (Rejano et al., 2010). Briefly, green olives were treated with a solution of NaOH (2–2.5% [w/v]) with the addition, only in the case of *patio* #1, of NaCl (15.3 g/L) and $CaCl_2$ (0.83 g/L), for 8–10 h; the olives were then washed with water to remove the excess of alkali and finally covered with brine (10–11% [w/v] NaCl). Again, only in the case of *patio* #1, brine contained 1.87 g/L $CaCl_2$. At this point, treated olives plus brine are used to fill up the 10-tonne fermenters located in the *patios*. Only in *patio* #1, brines were acidified by the addition of 25 L of food-grade HCl. After 1–2 months of fermentation, in both *patios ca.* 500 L of the fermenting brine taken from the bottom of the fermenters, and containing olive debris and more alkaline conditions, were discarded. The fermenters were then refilled with fresh brine containing lactic acid and HCl (usually 5 L each), being this a common practice in large table-olive manufacturing companies to avoid spoilage. Olives were all of the Manzanilla variety and no starter culture was used. Fermentations were set up during September 2010 and three consecutive 50-ml samples were taken from each fermenter at approximately monthly intervals, in coincidence with the initial, middle and final stages of green olives fermentation. As the harvesting of the fruits as well as the processing capacity of these industries had an obvious daily limitation, only a limited number of fermentations could be set up daily. Therefore, at each of the three sampling dates, brine samples collected from the fermenters at each *patio* felled into a range of time after brining. More specifically, fermentation had taken place for 1–14 (first two weeks), 35–48 (5th–7th week), and 69–72 (10th–12th week) days after brining, for sampling points #1, 2 and 3, respectively. Samples were added glycerol so that final concentration was 20% (v/v) and stored at −80 °C until use.

Table 1
Primers used in this study.

Primer	Sequence (5′–3′)	References
OPL5	ACGCAGGCAC	Maldonado-Barragán et al., 2013
ISS1rev	GGATCCAAGACAACGTTTCAAA	Veyrat et al., 1999
plb16	AGAGTTTGATCCTGGCTCAG	Kullen et al., 2000
mlb16	GGCTGCTGGCACGTAGTTAG	Kullen et al., 2000
paraF	GTCACAGGCATTACGAAAAC	Torriani et al., 2001
pentF	CAGTGGCGCGGTTGATATC	Torriani et al., 2001
planF	CCGTTTATGCGGAACACCTA	Torriani et al., 2001
pREV	TCGGGATTACCAAACATCAC	Torriani et al., 2001
PAR	GACGGTTAAGATTGGTGAC	Ventura et al., 2003
CAS	ACTGAAGGCGACAAGGA	Ventura et al., 2003
RHA	GCGTCAGGTTGGTGTTG	Ventura et al., 2003
CPR	CAANTGGATNGAACCTGGCTTT	Ventura et al., 2003
NL1	GCATATCAATAAGCGGAGGAAAAG	Kurtzman and Robnett, 1998
NL4	GGTCCGTGTTTCAAGACGG	Kurtzman and Robnett, 1998

2.2. Isolation of microorganisms

Aliquots of samples stored at −80 °C were defrost at room temperature, serially diluted in 0.1% (w/v) peptone water and extended in duplicates onto agar plates of culture media. Five different culture media were used in this study: Brain Heart Infusion (BHI; Biokar Diagnostics, Beauvais, France) supplemented with 0.05% L-cysteine (AppliChem, Darmstadt, Germany); de Man-Rogosa-Sharpe (MRS; Biokar Diagnostics) supplemented with 0.02 g/L bromophenol blue (AppliChem) and L-cysteine (MRS-BPB; Lee and Lee, 2008); Reinforced Clostridial Medium (RCM; Biokar Diagnostics); and MacConkey Broth Purple (Biokar Diagnostics). Seeded plates were incubated anaerobically at 30 °C for three days, except for RCM, when seven-day incubations were used. For anaerobic incubations we used a DG250 Anaerobic Workstation (Don Whitley Scientific Ltd., Shipley, West Yorkshire, UK), with a gas mixture consisting of 10% H_2-10% CO_2-80% N_2. Glucose–Yeast Extract Agar supplemented with oxytetracycline (0.1 g/L) (OGYE; Mossel et al., 1962) was incubated aerobically at 30 °C for 2 days. Agar was added to the broth media at 1.5% (w/v). Prior to spreading onto RCM agar plates, samples were pasteurised at 75 °C for 15 min in a water bath. For further studies, a single colony of each different morphotype identified in each culture medium at every sampling point was selected from plates with low counts, purified by repeated subculturing and observed under a phase-contrast microscope (Olympus Optical Co., Tokyo, Japan) to distinguish its cell

morphology. For long-term storage, purified isolates were preserved at −80 °C in their culture medium containing glycerol (20% v/v). All isolates were subjected to genotyping as described below.

2.3. Molecular identification techniques

Total DNA was extracted directly from colonies by the rapid chloroform method described by Ruiz-Barba et al. (2005). The same DNA extraction, preserved at 4 °C, was used for all subsequent molecular techniques. Primers used in this study are described in Table 1.

2.3.1. Genotyping by Randomly Amplified Polymorphic DNA (RAPD)

Microbial isolates were grouped by their cell morphology before strain typification by the RAPD fingerprinting technique. Genotyping was carried out by RAPD using the primer OPL5 as described by Maldonado-Barragán et al. (2013). In the case of coccus-shaped bacteria, primer ISS1rev was used instead. The resulting RAPD profiles were normalised and analysed for clustering with the Bionumeric 7.0 software package (Applied Maths, Sint-Martens-Latem, Belgium). Only bands representing amplicons between 150 and 5000 bp in size were included in the analysis. Similarity dendrograms were constructed by the UPGMA clustering method, using the band-based Dice similarity coefficient. Similarity coefficient ≥0.80 was considered as a cut-off value for isolates belonging to the same strain. A representative isolate of each RAPD profile was selected for further characterisation.

2.3.2. 16S rDNA sequence analysis of bacterial isolates

Representative bacterial isolates were identified to the genus level and/or to the species level by PCR sequencing of a *ca*. 500-bp fragment of the 16S rDNA gene, using the primer pair plb16/mlb16. PCR conditions were as described by Delgado et al. (2008). Briefly: initial denaturation at 96 °C for 30 s, followed by 30 cycles of denaturation at 96 °C for 30 s, annealing at 50 °C for 30 s, and polymerisation at 72 °C for 45 s, plus a final polymerisation step at 72 °C for 4 min. MyTaq DNA polymerase (Bioline, London, UK) was used according to the manufacturer instructions. The resulting amplicons were purified using a Nucleospin Extract II kit (Macherey–Nagel, Düren, Germany) and sequenced at Newbiotechnic S.A. (Bollullos de la Mitación, Spain). The resulting sequences were used to search for similarities in the relevant data banks using the Nucleotide BLAST utility at the NCBI web page (http://blast.ncbi.nlm.nih.gov/Blast.cgi) by limiting this search to type strains ("*Entrez query*" *option*). The identities of the representative isolates were determined on the basis of the highest scores (typically ≥98%).

2.3.3. PCR amplification with species-specific primers

Species-specific PCRs were performed for further discrimination when the results of 16S rDNA sequence analysis were not enough to identify species belonging to some bacterial groups. Species belonging to the *L. plantarum* group, i.e. *L. plantarum*, *L. pentosus* and *Lactobacillus paraplantarum*, were distinguished using a multiplex PCR assay with the *recA* gene-based primers paraF, pentF, planF and pREV as described by Torriani et al. (2001). Species belonging to the *Lactobacillus casei* group, i.e. *L. casei*, *Lactobacillus paracasei* and *Lactobacillus rhamnosus*, were distinguished using a multiplex PCR assay with the *tuf* gene-based primers CAS, PAR, RHA and CPR as described by Ventura et al. (2003).

2.3.4. 26S rDNA sequence analysis of yeast isolates

Representative yeast isolates were identified to the genus level and/or to the species level by PCR sequencing of the D1/D2 domain of the 26S *rDNA* gene (Kurtzman and Robnett, 1998). For this purpose, PCR amplification of the 26S rDNA gene using the universal primers NL1 and NL4 was performed as described by Kurtzman and Robnett (1998). The resulting amplicons were purified, sequenced and analysed according to the criteria for the differentiation of yeast species defined by Kurtzman and Robnett (1998), who considered a similarity higher than 99% to assign an isolate to a yeast species after doing a BLAST search in the relevant data banks.

2.4. Physico-chemical analyses

Titratable acidity, expressed as g/L lactic acid, combined acidity, expressed as Eq/L NaOH, and pH were measured using a Metrohm 670 Titroprocessor (Herisau, Switzerland). Salt concentration was determined by titration with $AgNO_3$ and expressed as % (w/v) NaCl.

2.5. Statistical analyses

Total counts of microorganisms were expressed as the mean values of colony forming units (CFU) per millilitre of brine based on duplicate analyses made for each sample, including the standard deviation (SD) of the mean. The resulting values were transformed to logarithmic values before statistical analyses were performed. U Mann–Whitney tests were applied to determine statistically significant differences between the microbial counts in both *patios* at each sampling point and for each culture media used. The fermentation-time effect on averaged microbial counts recovered from each culture media in both *patios* was tested using Friedman tests. These analyses were performed using the SPSS 21.0 statistical software (SPSS Inc., Chicago, USA).

2.6. Biodiversity analyses

Biodiversity was estimated through different alpha and beta indexes. Menhinick's index (I_{Mn}) was used to evaluate species richness. This index is based on the presumed linear relationship between the species richness and the total number of individuals. The Shannon–Weaver index (H') was used to estimate diversity and reflected the amount of disorder in the species distribution of the observed community. Evenness, or equitability, was measured through Pielou's index (J'). This index provided a sense of how evenly the different species contributed to the Shannon–Weaver diversity index. Simpson's reciprocal index ($1/D$) measured the number of equally common species that will produce an observed Simpson's index (D), which measures dominance. These alpha indexes were used to display the changes in the communities during fermentation, allowing also comparisons among them. They were calculated according to the following equations (Magurran, 2004):

$$I_{Mn} = S\Big/\sqrt{N} \tag{1}$$

$$\mathrm{H}' = -\sum p_i{*}\ln(p_i) \tag{2}$$

$$\mathrm{J}' = \mathrm{H}'/\ln S \tag{3}$$

$$1/\mathrm{D} = 1\Big/\sum p_i^2 \tag{4}$$

where p_i is the relative abundance of species i, S is the number of species present and N is the total number of individuals. Beta indexes were used to evaluate pairwise similarities between whole microbial communities, which were determined by calculating Jaccard's similarity coefficient (Sj) and Whittaker's index of

association (*Sw*) (Whittaker, 1952) using the following equations (Legendre and Legendre, 1998):

$$Sj = W/(a1 + a2 - W) \quad (5)$$

where W is the number of species shared between populations 1 and 2, while $a1$ and $a2$ are the total number of different species in populations 1 and 2, respectively;

$$Sw = 1 - \sum |b_i1 - b_i2| \Big/ 2 \quad (6)$$

where b1 and b2 are the percentage contributions of the *i*th species in samples 1 and 2, respectively. Both Jaccard (presence–absence) and Whittaker (proportional) indexes are measures of the similarity between communities (*patios*), with values from 0 (completely different) to 1 (identical). These indexes were used to compare changes in communities over time and between communities at each fermentation stage. Diversity indexes were calculated manually. Mean values of alpha diversity indexes among time periods were compared through the ANOVA of repeated measures in each community. Comparisons of mean values of alpha diversity indexes between communities were done by *t*-Student's test. Bartlett and Levene tests were used to check for homogeneity of the variance, while Kolmogorov–Smirnov test was used to check for normality. When it was necessary, values were transformed before the parametric test was carried out. To estimate diversity conservatively, singletons (species represented by just one individual) as well as unidentified microorganisms were removed prior to community analyses, as suggested by Zhou et al. (2013).

3. Results

3.1. Physico-chemical analyses

NaCl concentration in the brines reached an equilibrium during the first week of fermentation, showing values of 7.76 (±0.24) and 5.88 (±0.29) % (w/v) in the fermenters at *patio* #1 and #2, respectively. Values of pH evolved in a different manner in both *patios*, for in *patio* #1 brines were acidified since the beginning. In *patio* #1, pH values were 5.7 (±0.67), 4.0 (±0.1) and 3.91.0 (±0.12), while in *patio* #2, pH values were 7.43 (±0.53), 4.3 (±0.11) and 4.29 (±0.14) at the initial, middle and final fermentation stages, respectively. Titratable acidity at the final stage was 1.14 g/L (±0.08) and 0.78 g/L (±0.08), while combined acidity was 0.14 (±0.04) and 0.16 (±0.01) Eq/L for *patio* #1 and #2, respectively. All these parameters were considered normal for this table olive preparation.

3.2. Microbiological analyses

Averaged total counts of microorganisms isolated in the different culture media used in this study are shown in Table 2. Significant differences could be found between both *patios* in most culture media and fermentation stages. Higher count numbers were found in *patio* #2 in MRS-BPB, BHI and MacConkey in most cases. In contrast, higher counts were found in OGYE (mostly yeast) in *patio* #1 at the initial and middle fermentation stages. Nevertheless, total number of microorganisms isolated in MRS-BPB (mostly LAB) and OGYE were not significantly different at the final stage of the fermentation (Table 2). Microorganisms isolated in RCM at the middle and final stages of the fermentations were so scarce that no statistical tests could be properly carried out, although counts were very similar in both *patios* at every stage. Considering the dynamics of microbial populations along the time, significant differences in all culture media, except in OGYE, could be found in *patio* #2, while such time effect could only be detected in the evolution of microorganisms isolated in MRS-BPB and OGYE from *patio* #1.

Table 2
Averaged microbial counts along Spanish-style green-olive fermentations in two fermentation yards (*patios*) obtained in the culture media used in this study.

Culture medium	Fermentation stage	Patio 1	Patio 2	*P*-value[a]
MRS-BPB	Initial	3.78 (0.74)[b]	6.73 (0.69)	0.000
	Middle	5.78 (0.84)	7.33 (0.23)	0.000
	Final	6.19 (0.79)	6.42 (0.20)	NS[d]
Sig.[c]		*	*	
BHI	Initial	4.42 (0.91)	6.60 (0.70)	0.000
	Middle	5.34 (0.89)	7.38 (0.35)	0.000
	Final	5.46 (0.82)	6.37 (0.30)	0.006
Sig.			*	
MacConkey	Initial	4.18 (1.27)	2.06 (1.99)	0.015
	Middle	4.68 (1.26)	6.38 (0.44)	0.001
	Final	3.29 (2.12)	5.47 (0.41)	0.013
Sig.			*	
RCM	Initial	1.24 (0.86)	1.19 (1.12)	NS
	Middle	0.51 (0.82)	0.37 (0.78)	–[e]
	Final	0.34 (1.08)	0.17 (0.54)	–
Sig.		–	–	
OGYE	Initial	3.83 (0.43)	2.47 (1.25)	0.010
	Middle	3.56 (1,19)	2.10 (1.69)	0.045
	Final	2.62 (1.04)	2.74 (0.52)	NS
Sig.		*		

[a] Statistical significance considering both *patios* at each fermentation stage (U Mann–Whitney's test; for $P \leq 0.05$).
[b] Mean log CFU/ml (standard deviation), $n = 10$.
[c] Sig.: statistical significance of time effect in the fermentation within each *patio* (Friedman-test; * for $P < 0,05$).
[d] NS, not significant difference.
[e] –, not enough data to carry out the statistical test.

3.3. Bacterial diversity and dynamics

Bacterial species isolated as well as the number of isolates along the Spanish-style green olive fermentation in two different *patios* are shown in Table 3, where they are arranged regarding their abundance. Also, the relative abundance of bacterial species found in each of the 20 fermenters under study, at the three fermentation stages considered, is shown in Fig. 1. A total of 37 different species were found, belonging to 18 different bacterial genera. The vast majority were Gram positive bacteria, i.e. 76% and 80% in *patio* #1 and #2, respectively. It was noteworthy the ubiquitous presence of the species *L. pentosus* in all 20 fermenters under study at virtually every sampling point (Fig. 1). Seven other species could be also found in both *patios*, i.e. *Lactobacillus paracollinoides/collinoides*, *Lactobacillus parafarraginis*, *Lactobacillus rapi*, *Pediococcus ethanolidurans*, *Staphilococcus* sp., *Pediococcus parvulus* and *Paenibacillus illinoisensis/xylanilyticus* (Table 3). In addition, all these species, except *P. illinoisensis/xylanilyticus*, were isolated at the same fermentation stages from both *patios*, and especially at the final stage (Table 3). With up to16 species found, it is remarkable the prevalence of LAB in both *patios*: *ca.* 92% and 97% of the isolates in *patio* #1 and #2, respectively, and 72% and 93%, respectively, when removing the *L. pentosus* isolates. The maximum number of distinct species was found at the initial stage of fermentation, so that 22 out of the 37 bacterial species found were isolated only at this occasion, 13 of them from *patio* #1 and 8 of them from *patio* #2, being *L. pentosus* the only common species at this stage. Nevertheless, many of the species which were only isolated at the first stage could only be detected in one or two of the fermenters in each *patio*. The exceptions were the species *Enterococcus casseliflavus*, *Vibrio furnissii/fluvialis* and *Weisella*

Table 3
Bacterial species isolated along Spanish-style green olive fermentations in two different fermentation yards ("*patios*").

Bacterial species	Fermentation stage			Total[a] isolates	No.[b] ferm.	Count range[c] (log CFU/ml)	References[d] Spanish-style	Other
	Initial	Middle	Final					
Patio 1								
Lactobacillus pentosus	74[e]	98	135	307	10	1–6	a – f	d, e, g – n
Lactobacillus paracollinoides/collinoides	0	20	13	33	8	1–5	f	i, l, o
Pediococcus ethanolidurans	0	3	18	21	4	1–5	f	k
Enterococcus casseliflavus	11	0	0	11	5	1–2	a	h
Lactobacillus parafarraginis	0	4	6	10	7	1–5	f	
Vibrio furnissii/fluvialis[f]	9	0	0	9	6	2–3		
Staphylococcus sp.[g]	3	2	2	7	6	1–5		
Weissella paramesenteroides/hellenica	7	0	0	7	6	1		h
Lactobacillus plantarum	5	0	0	5	2	1–3	d, e, p–r	d, g, h, k, l, n, o, s, t
Pediococcus parvulus	0	0	4	4	2	3–5		j, l, m
Clostridium xylanovorans	3	1	0	4	4	1		
Propionibacterium acnes	0	0	3	3	1	4	u	
Escherichia sp.[h]	2	0	0	2	1	1	v, w	
Lactobacillus rapi	0	0	1	1	1	3	f	
Pantoea agglomerans[m]	0	0	1	1	1	3		i
Bacillus circulans[m]	1	0	0	1	1	1		
Bacillus weihenstephanensis/mycoides[m]	0	1	0	1	1	1		
Brachybacterium muris[m]	0	1	0	1	1	1		
Clostridium jejuense[m]	1	0	0	1	1	1		
Clostridium sartagoforme[m]	1	0	0	1	1	1		
Clostridium schirmacherense/argentinense[m]	1	0	0	1	1	1	x	x
Enterobacter hormaechei[m]	1	0	0	1	1	1		
Enterobacter radicincitas/oryzae[m]	1	0	0	1	1	1		
Enterobacter sp[i,m]	1	0	0	1	1	1	v, w, y	
Paenibacillus illinoisensis/xylanilyticus	0	1	0	1	1	1		
Total isolates[j]	121	131	183	435[k]				
Species richness	15	9	9	25				
Species richhness w/o singletons	8	7	8	15				
Patio 2								
Lactobacillus pentosus	48[e]	109	168	325	10	1–7	a–f	d, e, g–n
Aerococcus viridans/urinaeequi	55	0	0	55	9	1–5	z	
Pediococcus parvulus	0	15	19	34	10	4–6		j, l, m
Lactobacillus paracasei	0	18	2	20	7	3–7	d	h, l, n, s, t
Enterococcus saccharolyticus[f]	16	0	0	16	10	2–6		
Lactobacillus coryniformis	0	4	6	10	5	4–6	b	h, k, l
Lactobacillus rhamnosus	0	2	4	6	3	4–5		h, s, t
Staphylococcus sp.[g]	0	1	5	6	5	1–5		
Lactobacillus rapi	0	3	2	5	4	4–6	f	
Lactobacillus paracollinoides/collinoides	0	0	4	4	4	4–5	f	i, l, o
Pediococcus ethanolidurans	0	0	2	2	1	4	f	k
Paenibacillus sp.[l]	2	0	0	2	2	1		
Sporolactobacillus inulinus/terrae	0	2	0	2	1	1		
Lactobacillus parafarraginis	0	0	1	1	1	4	f	
Lactobacillus paraplantarum[m]	1	0	0	1	1	3	e	d, g, k, n
Enterobacter kobei[m]	1	0	0	1	1	2		
Escherichia coli[m]	1	0	0	1	1	2	v, w	
Paracoccus carotinifaciens[m]	1	0	0	1	1	2		
Paenibacillus illinoisensis/xylanilyticus	1	0	0	1	1	1		
Yersinia enterocolitica[m]	1	0	0	1	1	1		
Total isolates[j]	127	154	213	494[k]				
Species richness	10	8	10	20				
Species richness w/o singletons	5	8	10	15				

Key to references: a, De Castro et al., 2002; b, Aponte et al., 2012; c, Ruiz-Barba and Jiménez-Díaz, 2012; d, Doulgeraki et al., 2013; e, Bautista-Gallego et al., 2013; f, Montaño et al., 2013; g, Hurtado et al., 2008; h, De Bellis et al., 2010; i, Abriouel et al., 2011; j, Franzetti et al., 2011; k, Doulgeraki et al., 2012; l, Randazzo et al., 2012; m, Abriouel et al., 2012; n, Argyri et al., 2013; o, Chamkha et al., 2008; p, Ruiz-Barba et al., 1991; q, Ruiz-Barba et al., 1994; r, Ruiz-Barba and Jiménez-Díaz, 1995; s, Balloni et al., 1973; t, Mourad and Nour-Eddine, 2006; u, González-Cancho et al., 1980; v, Borbolla y Alcalá et al., 1960; w, González Cancho, 1963; x, Pereira et al., 2008; y, Bevilacqua et al., 2010; z, González-Cancho and Durán-Quintana, 1981.

[a] Total isolates of a specific bacterial species.
[b] Number of fermentors, out of a total of ten, from which a specific bacterial species was isolated in each *patio*.
[c] Colony count range at which that bacterial species was isolated.
[d] Bibliographic reference which cited that particular bacterial species in Spanish-style and/or other table olive preparations.
[e] Number of isolates of that bacterial species at that sample point.
[f] The relatively low (≤97%) 16S rDNA homology of these isolates with other bacterial species in the data banks could indicate that they might be novel species.
[g] The most homologous species were *Staphilococcus epidermidis, S. saccharoliticus, S. capitis* and *S. caprae*.
[h] The most homologous species were *Escherichia coli, E. senegalensis* and *E. fergusonii.*
[i] The most homologous species were *Enterobacter cloacae, E. sacchari, E. kobei* and *E. radicincitas*.
[j] Total bacterial isolates at each sampling point.
[k] Total bacterial isolates in each *patio*.
[l] The most homologous species were *Paenibacillus taichungensis*, *P.tundrae*, *P. tylopili*, and *P. barcinonensis*, *P.amylolyticus*.
[m] Species which have been considered singletons and have been removed from the diversity analyses.

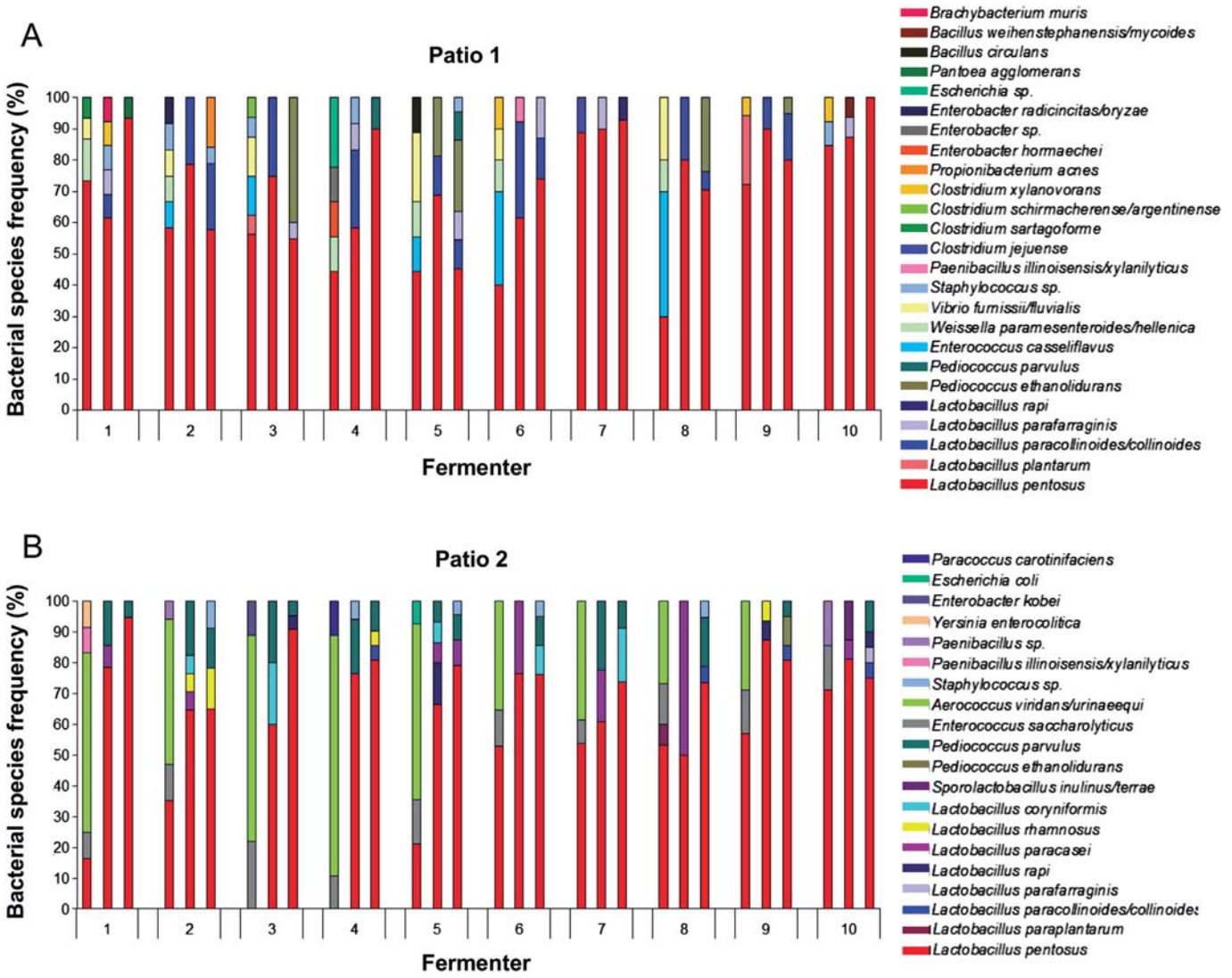

Fig. 1. Bacterial species frequency in ten fermenters of the fermentation yard (*patio*) #1 (panel A) and #2 (panel B). For each fermenter, from left to right, the three bars represent the bacterial species frequency at the initial, middle and late stages of fermentation, respectively.

paramesenteroides/*hellenica* in *patio* #1, and *Aerococcus viridans*/*urinaeequi* and *Enterococcus saccharolyticus* in *patio* #2, which were isolated from most fermenters at each *patio* (Table 3). In contrast, a few species could be detected only at the final stage of fermentation: *Pantoea agglomerans* in *patio* #1, and *L. paracollinoides*/*collinoides*, *P. ethanolidurans* and *L. parafarraginis* in *patio* #2. While only *L. pentosus* could be isolated from all of the fermenters in *patio* #1, *P. parvulus* and *E. saccharolyticus*, apart from *L. pentosus*, were isolated from all fermenters in *patio* #2. Other species which were isolated from 6 or more fermenters at each *patio* were *L. paracollinoides*/*colinoides*, *L. parafarraginis*, *Vibrio furnisii*/*fluvialis*, *Staphilococcus* sp. and *W. paramesenteroides*/*hellenica* in *patio* #1, and *A. viridans*/*urinaeequi* and *L. paracasei* in *patio* #2

Regarding the counts of each species, with the exception of *A. viridans*/*urinaeequi* and *E. saccharolyticus* in *patio* #2, those which reached the largest concentrations in the brines (more than 10^5 CFU/ml) were isolated at the middle and final stages of fermentation (Table 3). Again, the prevalence of the species *L. pentosus* was clear but other species reached high count numbers. This was especially true for most LAB species (lactobacilli, pediococci and enterococci), but also for *Staphylococcus* sp. (Table 3). Species belonging to the enterobacteriaceae group were all isolated only at the initial stage, except for *P. agglomerans* at the final stage, being their counts as well as the number of fermenters colonised by this group extremely low.

Authors that, to our knowledge, have cited the isolation or DNA amplification of any of the bacterial species found in this study, either in Spanish-style or any other table olive preparations, are referenced in Table 3. A total of 20 bacterial species, i.e. more than 50%, have been isolated for the first time from Spanish-style green olive fermentations in this study, while 17 had not been described before in any table olive preparation. The genera *Brachybacterium*, *Paenibacillus*, *Sporolactobacillus*, *Paracoccus* and *Yersinia* had not been cited before from any table olive preparation to our knowledge.

3.4. Yeast diversity and dynamics

Yeast species isolated along the Spanish-style green olive fermentation in the two *patios* of this study, arranged according to their abundance, are shown in Table 4. The relative abundance of

Table 4
Yeast species isolated along Spanish-style green olive fermentations in two different fermentation yards ("*patios*").

Yeast species	Fermentation stage			Total[a] isolates	No. ferm[b]	Count range[c] (log CFU/ml)	References[d] Spanish-style	Other
	Initial	Middle	Final					
Patio 1								
Saccharomyces cerevisiae	2[e]	12	3	17	9	1–5	a–e	e–q
Issatchenkia orientalis	17	0	0	17	8	1–2	a–c, m, r	n, q, s, t
Candida tropicalis	12	0	0	12	6	1–2	a, c, d, m	m
Candida thaimueangensis	1	4	7	10	7	1–2	m	m
Candida butyri/aaseri	9	0	0	9	6	1–2		j, n, u
Rhodotorula mucilaginosa	0	0	4	4	4	1		j, n, p, v, w
Saturnispora mendoncae	3	0	0	3	3	2		
Hanseniaspora sp.[f]	3	0	0	3	3	1–2	m	g, l
Candida parapsilosis[j]	0	0	1	1	1	1	a, b, d, r	h, i, u
Other yeast sp[g]	21	3	0	24	9	1–4		
Total isolates[h]	68	19	15	102[i]				
Species richness	8	3	4	10				
Species richness w/o singletons	8	3	3	9				
Patio 2								
Candida thaimueangensis	0	2	17	19	10	1–2	m	m
Saccharomyces cerevisiae	8	2	0	10	8	1–4	a–e	e–q
Kluyveromyces lactis/marxianus	0	4	0	4	4	1	m	i, u
Pichia manshurica/membranifaciens	0	1	3	4	4	1	a–d, m	j–o, q, s, t, w–z
Hanseniaspora sp.[f]	1	0	0	1	1	1–4	m	g, l
Candida glabrata[j]	1	0	0	1	1	1	a, c	i, v
Total isolates[h]	10	9	20	39[i]				
Species richness	3	4	2	6				
Species richness w/o singletons	2	4	2	5				

Key to references: a, González Cancho, 1963; b, González-Cancho, 1965; c, González Cancho, 1966a; d, González Cancho, 1966b; e, Garrido Fernández et al., 1997; f, Marquina et al., 1992; g, Arroyo-López et al., 2006; h, Mourad and Nour-Eddine, 2006; i, Hernández et al., 2007; j, Nisiotou et al., 2010; k, Rodríguez-Gómez et al., 2010; l, Silva et al., 2011; m, Bautista-Gallego et al., 2011; n, Muccilli et al., 2011; o, Abriouel et al., 2011; p, Alves et al., 2012; q, Golomb et al., 2013; r, Mrak et al., 1956; s, González-Cancho et al., 1975; t, Doulgeraki et al., 2012; u, Hurtado et al., 2008; v, Campaniello et al., 2005; w, Franzetti et al., 2011; x, Oliveira et al., 2004; y, Coton et al., 2006; z, Chamkha et al., 2008.

[a] Total isolates of a specific yeast species.
[b] Number of fermentors, out of a total of ten, from which a specific yeast species was isolated in each *Patio*.
[c] Colony count range at which that yeast species was isolated.
[d] Bibliographic reference which cited that particular yeast species in Spanish-style and/or other table olive preparations.
[e] Number of isolates of that yeast species at that sample point.
[f] The most homologous species were *Hanseniaspora opuntiae*, *H. meyeri*, *H. lachancei* and *H. uvarum*.
[g] These yeast isolates could not be adscribed to any specific yeast species.
[h] Total yeast isolates at each sampling point.
[i] Total yeast isolates in each *Patio*.
[j] Species which have been considered singletons and have been removed from the diversity analyses.

yeast species found in each of the 20 fermenters under study, at the three fermentation stages considered, is shown in Fig. 2. Taking into account that 24 isolates from *patio* #1 could not be assigned to any specific species with a minimum of confidence, a total of 12 different species were found, belonging to 7 different yeast genera. More yeast isolates and species diversity was found in *patio* #1 than in *patio* #2, especially at the initial fermentation stage (Table 4). Three yeast species could be isolated from both *patios*, i.e. *Saccharomyces cerevisiae*, *Candida thaimueangensis* and *Hanseniaspora* sp., being also detected at similar fermentation stages. In contrast with the results obtained for bacteria, two yeast species appeared to somehow dominate the yeast microbiota: *C. thaimueangensis* and *S. cerevisiae* (Table 4). These two species were isolated from most fermenters in both *patios* at most fermentation stages. *S. cerevisiae* appeared to be dominant at the initial and middle stages, while *C. thaimueangensis* increased its presence as fermentation progressed and dominated the final stage. Regarding their relative abundance, counts were especially high for *Saccharomyces* sp. (Table 4). *Issatchenkia orientalis* and different species of *Candida* were also very abundant in most fermenters of *patio* #1, where they were isolated mostly at the first fermentation stage (Table 4). As for bacteria, authors that have cited the isolation or DNA amplification of any of the yeast species found in this study are referenced in Table 4. To our knowledge, the species *Candida butyri/asseri* and *Rhodotorula mucilaginosa* had not been described before from Spanish-style green olive fermentations, while the species *Saturnispora mendoncae* had not been cited before from any table olive preparation (Table 4).

3.5. Biodiversity analyses

3.5.1. Alpha diversity indexes

Total bacterial species richness found was identical in each *patio* once singletons were removed, i.e. 15 species (Table 3), although this figure was lower when looking at each of the three fermentation stages considered (Table 3) or a particular fermenter (Fig. 1). Species richness was evaluated through the Menhinick's diversity index (I_{Mn}), which is shown in Fig. 3 (panel A). This index showed a decrease in its values as fermentation progressed in *patio* #1, with statistically significant differences between the initial and final stages of fermentation (Fig. 3). No significative differences, though, were found in *patio* #2 or between both *patios* at any fermentation stage. Bacterial diversity, evaluated by the Shannon–Weaver index (H'), is shown in Fig. 3 (panel B). Although the values of this index became lower as fermentation proceeded, no significative difference was found neither through the fermentation stages in any *patio* nor between both *patios*. Maximum values were always reached at the initial stage. The decrease in the values of the H' index in *patio* #2 can be explained by a parallel decrease of evenness in the distribution of the species found, as indicated by the Pielou's index (J') (Fig. 3, panel C).

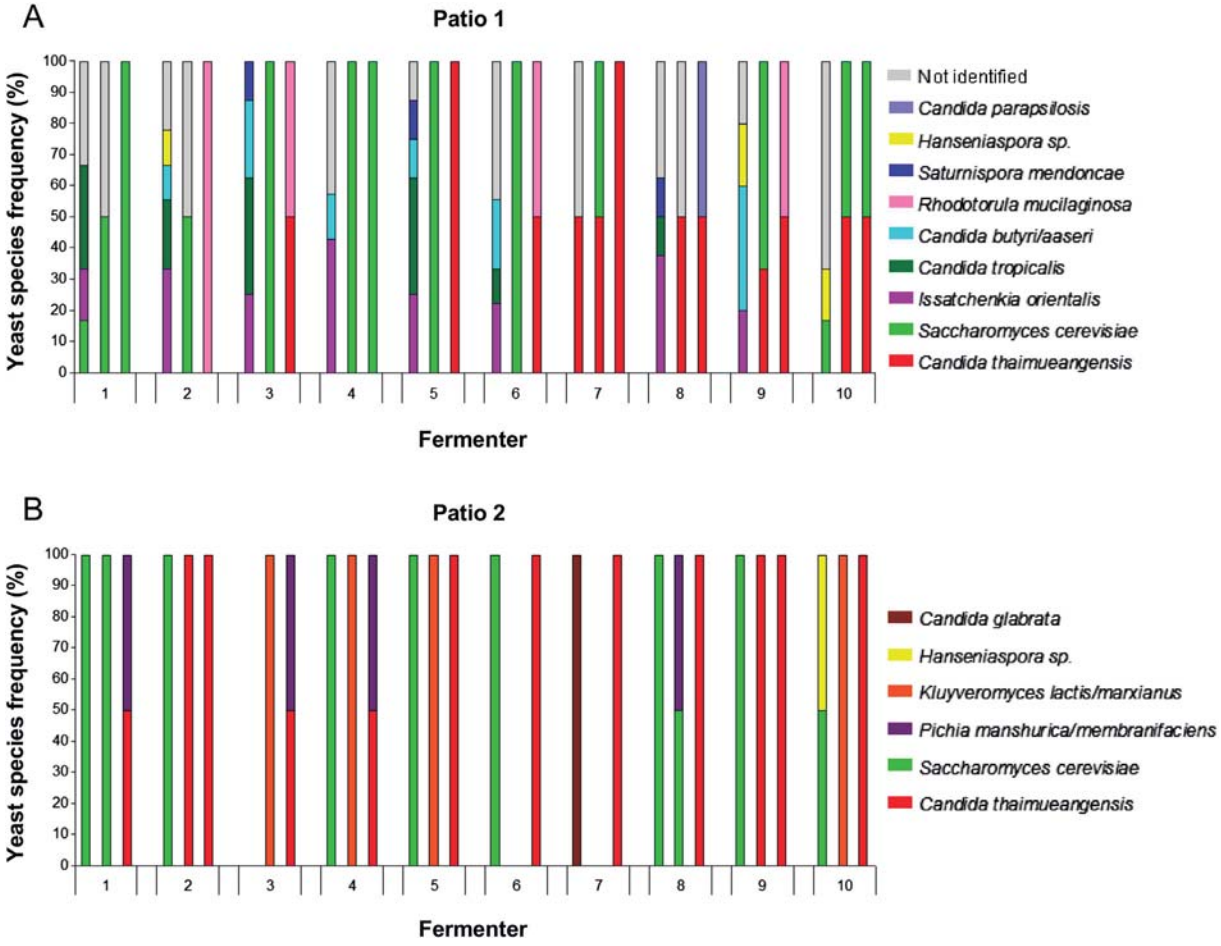

Fig. 2. Yeast species frequency in ten fermenters of the fermentation yard (*patio*) #1 (panel A) and #2 (panel B). For each fermenter, from left to right, the three bars represent the yeast species frequency at the initial, middle and late stages of fermentation, respectively.

Actually, the difference of evenness is statistically significant between the initial and final stages of fermentation in *patio* #2. Dominance, as expressed by Simpson's reciprocal index (1/*D*) (Fig. 3, panel D), followed a pattern similar to bacterial diversity evaluated trough the Shannon–Weaver index (Fig. 3, panel B). Again, no significant difference was found between both *patios* at any stage of the fermentation. However, a significant difference could be found between the initial and final stages in *patio* #2. This is due to the fact that Simpson's reciprocal index put more weight on most abundant species, being more influenced by the values of evenness indexes than those of species richness. This explains the fact that, although there is an increase in species richness in *patio* #2 along fermentation time, these species are less evenly distributed, producing a statistically significant change in the nature of dominant species. In contrast, in *patio* #1 evenness is quite similar across the three fermentation stages so that the decrease in the values of Simpson's reciprocal index is again due to loss of species richness.

Yeast species richness was quite lower than bacterial one, being also quite different in the two *patios* under study (Table 4). The low number of species isolated when sampling any fermenter along the fermentation time made advisable to calculate diversity indexes globally for each fermenter, i.e. not considering the fermentation stages. The values of these indexes are shown in Fig. 4. Significative differences between both *patios* were found in the values for the Shannon-Weaver's (H'), Pielou's (J') and Simpson's reciprocal ($1/D$) indexes, being these values always higher in *patio* #1 (Fig. 4). However, no significant difference was found regarding species richness estimated through Menhinick's index (I_{Mn}). This is the result of an unequal amount of sampling effort in both patios, for yeast counts on OGYE medium were significantly lower (Table 2) and its species composition less diverse (Table 4) in *patio* #2 at the initial and middle fermentation stages.

3.5.2. Beta diversity indexes

Pair-wise comparisons of microbial community composition using Jaccard and Whittaker beta diversity indexes for bacteria and yeast are shown in Table 5. Regarding bacteria, the similarity between both communities, i.e. *patio* #1 and #2, became higher as fermentation went on from the initial to the final fermentation stages. Values obtained for Jaccard's coefficient were always lower than those for Whittaker's index, indicating that species shared by both *patios* were also the most abundant. This was supported by the fact that the species *L. pentosus* actually dominated all along the fermentation in both *patios* (Table 3 and Fig. 1). In addition, Jaccard's coefficient allowed us to perceive the ecological succession in the species structure of each community (*patio*) over time. Changes in the species composition were gradual, being more similar this composition at the middle and final fermentation stages. Finally, Whittaker's index values were higher for *patio* #1 than for *patio* #2, a result that is a consequence of a change in the species distribution in *patio* #2 between the initial and middle stages of the fermentation. More specifically, *L. pentosus* is the co-dominant species together with *A. viridans*/*urinaeequi* during the initial stage of

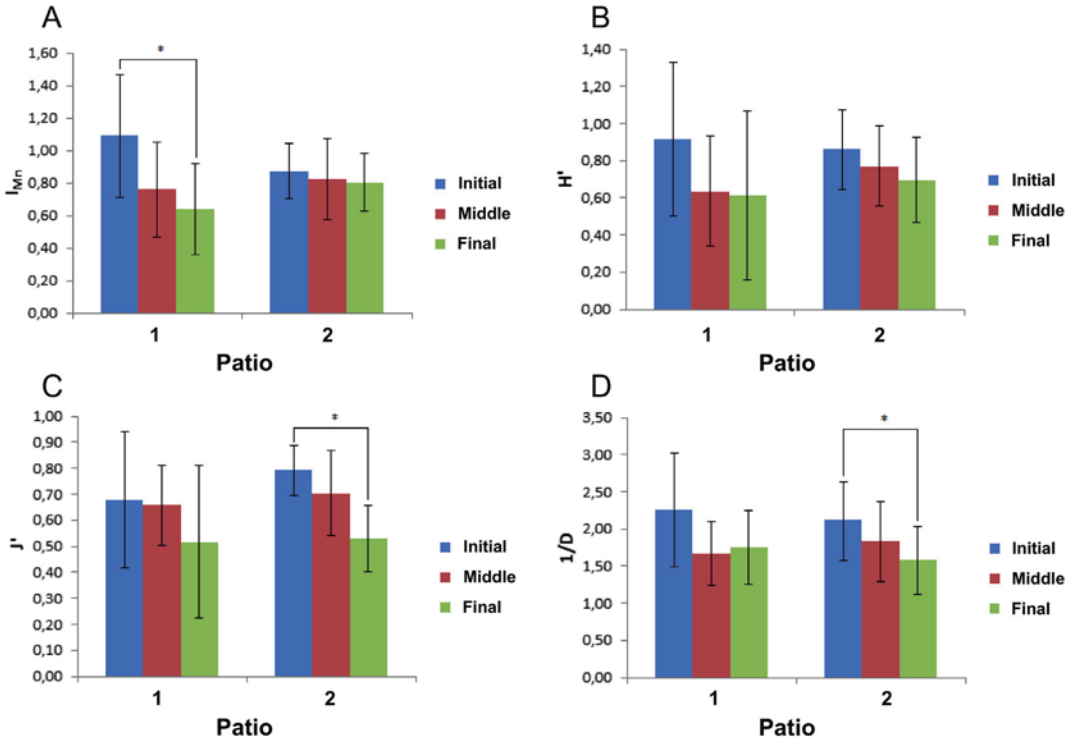

Fig. 3. Diversity indexes for bacteria in two fermentation yards (*patios*) along the three (initial, middle and final) stages of Spanish-style green olive fermentation. Panel A: Menhinick's diversity index (I_{Mn}); panel B: Shannon–Weaver's diversity index (H′); panel C: Pielou's evenness index (J′); panel D: Simpson's reciprocal index (1/D). Ten fermenters were studied at each *patio* ($n = 10$); bars indicate standard errors; * indicates a significant difference ($p < 0.05$). Singleton species have been removed from the analyses.

fermentation in this *patio*, while *L. pentosus* alone is the dominant species for the rest of the fermentation (Table 3 and Fig. 1).

In contrast to the results showed by the bacterial community, maximum similarity for yeast community composition between both *patios* was found at the middle stage of fermentation (Table 5). As for bacteria, an ecological succession was also observed over time, with a species composition more similar at the middle and final stages of fermentation. In the case of *patio* #2 the change in the yeast species composition is complete between the initial and final stages, as denoted by the 0.00 value for both Jaccard's and Whittaker's indexes (Table 5).

4. Discussion

The aim of this study was to update our knowledge on the microbiota associated to table-olive fermentations produced through the Spanish-style procedure. To accomplish this task we have used both, classic microbiological (culture dependent) techniques and modern molecular techniques for the identification of the different bacterial and yeast species isolated. In addition, our goal was not only to describe this microbiota and its evolution (dynamics) along the olive fermentations, but also to recover the microbial diversity associated to this traditional food fermentation as well as to preserve it for further biotechnological purposes. For this reason, our sampling strategy included the recovery of all morphological types appearing in the different culture media used, instead of the more usual "random" picking of the isolated colonies. In this sense, it was of the greatest value the use of a modified MRS-agar culture medium which included bromophenol blue as a discriminating agent of the actual metabolism/morphology of the isolates growing onto this medium, as proposed by Lee and Lee (2008) (see an example in the Supplementary Fig. S1).

Spanish-style green olive fermentations appeared to be dominated by the species *L. pentosus*. This observation is not novel, for other authors have reported this fact previously (De Castro et al., 2002; Ruiz-Barba and Jiménez-Díaz, 2012; Hurtado et al., 2012; Heperkan, 2013; among others). Furthermore, it is remarkable the ubiquitous presence of this species in all fermenters, at medium–high counts, since very early at the first stage of the fermentation. Therefore, it is not surprising that 68% of the total isolates belonged to this species. Apart from *L. pentosus*, a remarkably high number of LAB species, 15 in total, were isolated. Some of these species had not been described before from Spanish-style table-olive fermentations, i.e. *W. paramesenteroides/hellenica*, *P. parvulus*, *E. saccharolyticus*, *L. rhamnosus* and *Sporolactobacillus inulinus/terrae*. Actually, two of these species, i.e. *E. saccharolyticus* and *S. inulinus/terrae*, had not been cited before from any table-olive preparation. As many of these LAB species have been described as exerting some probiotic effect (Fontana et al., 2013), Spanish-style olive fermentation brines were revealed as a valuable source of potentially probiotic strains. In addition, *Enterococcus* species appeared to have a role at the crucial initial stage, with *E. casseliflavus* and *E. saccharolyticus* in *patio* #1 and #2, respectively. This observation was not novel, for actually De Castro et al. (2002) described the use of *E. casseliflavus* and *L. pentosus* as mixed starter cultures for Spanish-style green olive fermentation. Such use was based on the high-pH tolerance of *Enterococcus* species as well as its LAB character. Finally, two quite abundant and ubiquitous bacterial species were isolated at the initial stage of the fermentation whose 16S DNA showed similarity to the species *V. furnisii/fluvialis* and *E. saccharolyticus*, respectively. However, the percentages of similarity (≤97%) of the 16S DNA amplicon studied here, as well as other phenotypic (sugar metabolism) and genetic characteristics (DNA–DNA similarity) which have been investigated so far, suggested that these could constitute two novel species. Current efforts in our laboratory are focused on this purpose.

Yeast species were less abundant than bacteria, both in counts and number of species. Two yeast species appeared to be inherent

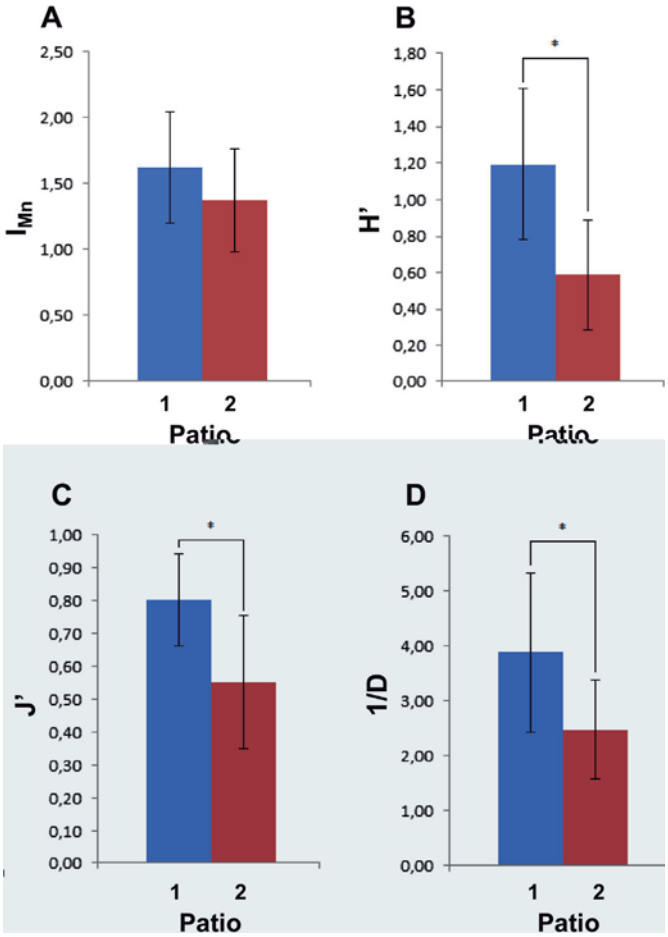

Fig. 4. Diversity indexes for yeast in two fermentation yards (*patios*) processing Spanish-style green olives. Panel A: Menhinick's diversity index (I_{Mn}); panel B: Shannon–Weaver's diversity index (H′); panel C: Pielou's evenness index (J′); panel D: Simpson's reciprocal index (1/D). Ten fermenters were studied at each *patio* ($n = 10$); bars indicate standard errors; * indicates a significant difference ($p < 0.05$). Singleton species and unidentified yeast have been removed from the analyses.

Table 5
Pair-wise comparisons of microbial community composition values in Spanish-style green olive fermentations using Jaccard and Whittaker beta diversity indexes.

Pair-wise comparisons		Beta diversity indexes			
		Bacteria		Yeast	
Patio	Fermentation stage	*Sj*[a]	*Sw*[b]	*Sj*	*Sw*
1	Initial/Middle	0.25	0.67	0.29	0.06
1	Middle/Final	0.50	0.88	0.67	0.46
1	Initial/Final	0.14	0.66	0.25	0.06
2	Initial/Middle	0.08	0.39	0.20	0.22
2	Middle/Final	0.64	0.86	0.50	0.33
2	Initial/Final	0.07	0.39	0.00	0.00
1/2[c]	Initial	0.08	0.39	0.29	0.11
1/2	Middle	0.15	0.71	0.50	0.44
1/2	Final	0.64	0.81	0.25	0.50

[a] Jaccard's coefficient.
[b] Whittaker's index of association.
[c] Comparison of the community composition between both patios at the different fermentation stages.

to the Spanish-style green olive fermentation in both *patios*, i.e. *S. cerevisiae* and *C. thaimueangensis*. In a recent study on the yeast diversity of table-olive fermentations, Bautista-Gallego et al. (2011) described the species *Candida tropicalis* and *Pichia galeiformis* as dominant in Spanish-style Manzanilla-variety olive fermentations in a manufacturing company which is, actually, geographically quite close to *patio* #1 studied here. Although these authors did not find *S. cerevisiae*, they described the isolation of *C. thaimueangensis*, but restricted just to the final fermentation stage while obtaining low number of isolates (12% of the yeast isolates at that stage). As for bacteria, it is very interesting to find yeast species not cited before either in Spanish-style, i.e. *C. butyri*/*asseri* and *R. mucilaginosa*, or in any table-olive preparation, as it is the case with *S. mendoncae*. This fact reinforces the idea of table-olive brines as a source of novel yeast strains with desirable biotechnological properties.

After evaluating microbial diversity through different alpha and beta indexes, our results showed again that this food fermentation was dominated by a single species, i.e. *L. pentosus*. Therefore, it was not unexpected that the actual values of different diversity indexes were relatively low, especially when singletons were removed from the analyses. For bacteria, although not always significative differences could be found, maximum diversity was displayed at the initial fermentation stage. Evenness, i.e. the frequency distribution of the different species, also decreased along the fermentation. This was due to the dominance exerted by the species *L. pentosus*, although statistically significant differences could only be observed in *patio* #2. This effect was most probably due to the change in the dominant species from the initial stage, i.e. *A. viridans*/*urinaeequi*, to the middle and final stages, dominated by *L. pentosus*. No significant differences could be found in any diversity index between both *patios* at any fermentation stage, suggesting that the process is quite "robust" once properly started. Although diversity was very similar in both *patios*, differences could be found in the actual composition of the "accessory" microbiota, i.e. that accompanying *L. pentosus* species. Nevertheless, most of this "accessory" microbiota was composed of other LAB and could represent a sort of "watermark" of a particular *patio*. Similar studies on consecutive olive fermenting seasons at the same *patios* could prove or discard such a hypothesis. On the other hand, yeast diversity was much lower than bacterial one, with significative differences between both *patios*. Diversity, evenness and dominance indexes were all higher in *patio* #1. This fact did not appear to have an effect on the outcome of the fermentation, estimated through the physical and chemical analyses used in this study. As other authors have described different yeast species compositions (Bautista-Gallego et al., 2011), especially regarding the dominant species, no critical role could be predicted for this microbial group in Spanish-style olive fermentation apart from its not-yet demonstrated, but suggested, influence on the organoleptic properties of the product (Arroyo-López et al., 2008).

We believe that this microbiological study is quite representative of the Spanish-style green olive fermentation because of the selection of two large, well-established and traditional table-olive manufacturing companies in the geographical area of maximum world production. In addition, the number and capacity of the fermenters from which samples were obtained, twenty 10-tonne fermenters representing *ca.* 200 tonnes of fermenting table olives, contributed to consistent and comprehensive results which will no doubt update our knowledge on this important food fermentation.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2014.03.020.

References

AAO, 2013. Agencia para el Aceite de Oliva. Olive Oil Agency. Market Information. Available at: http://aplicaciones.magrama.es/pwAgenciaAO/InfGeneral.aao?opcion_seleccionada=1000&control_acceso=S&idioma=ING (accessed 06.14.13.).

Abriouel, H., Benomar, N., Lucas, R., Gálvez, A., 2011. Culture-independent study of the diversity of microbial populations in brines during fermentation of naturally-fermented Aloreña green table olives. Int. J. Food Microbiol. 144, 487–496.

Abriouel, H., Benomar, N., Cobo, A., Caballero, N., Fernández Fuentes, M.A., Pérez-Pulido, R., Gálvez, A., 2012. Characterization of lactic acid bacteria from naturally-fermented Manzanilla Aloreña green table olives. Food Microbiol. 32, 308–316.

Alves, M., Gonçalves, T., Quintas, T., 2012. Microbial quality and yeast population dynamics in cracked green table olives' fermentations. Food Control 23, 363–368.

Aponte, M., Blaiotta, G., La Croce, F., Mazzaglia, A., Farina, V., Settanni, L., Moschetti, G., 2012. Use of selected autochthonous lactic acid bacteria for Spanish-style table olive fermentation. Food Microbiol. 30, 8–16.

Argyri, A.A., Zoumpopoulou, G., Karatzas, K.-A.G., Tsakalidou, E., Nychas, G.-J.E., Panagou, E.Z., Tassou, C.C., 2013. Selection of potential probiotic lactic acid bacteria from fermented olives by in vitro tests. Food Microbiol. 33, 282–291.

Arroyo-López, F.N., Durán-Quintana, M.C., Ruiz-Barba, J.L., Querol, A., Garrido-Fernández, A., 2006. Use of molecular methods for the identification of yeast associated with table olives. Food Microbiol. 23, 791–796.

Arroyo-López, F.N., Querol, A., Bautista-Gallego, J., Garrido-Fernández, A., 2008. Role of yeasts in table olive production. Int. J. Food Microbiol. 128, 189–196.

Balloni, W., Brighigna, A., Cucurachi, A., Florenzano, G., 1973. Prime ricerche sulla microflora lattica di alcune varietà italiana di olive da tavola e delle relative salamoie, vol. III. Annali dell'Istituto Sperimentale di Elaiotecnica, pp. 107–141.

Bautista-Gallego, J., Rodríguez-Gómez, F., Barrio, E., Querol, A., Garrido-Fernández, A., Arroyo-López, F.N., 2011. Exploring the yeast biodiversity of green table olive industrial fermentations for technological applications. Int. J. Food Microbiol. 147, 89–96.

Bautista-Gallego, J., Arroyo-López, F.N., Rantsiou, K., Jiménez-Díaz, R., Garrido-Fernández, A., Cocolin, L., 2013. Screening of lactic acid bacteria isolated from fermented table olives with probiotic potential. Food Res. Int. 50, 135–142.

Bevilacqua, A., Cannarsi, M., Gallo, M., Sinigaglia, M., Corbo, M.R., 2010. Characterization and implications of *Enterobacter cloacae* strains, isolated from Italian table olives "Bella di Cerignola". J. Food Sci. 75, 53–60.

Borbolla y Alcalá, J.M.R., Fernández-Díez, M.J., González-Cancho, F., 1960. Estudios sobre el aderezo de aceitunas verdes.XIX. Nuevas experiencias sobre el alambrado. Grasas Aceites 11, 256–260.

Botta, C., Cocolin, L., 2012. Microbial dynamics and biodiversity in table olive fermentation: culture-dependent and -independent approaches. Front. Microbiol. 3, 245.

Campaniello, D., Bevilacqua, A., D'Amato, D., Corbo, M.R., Altieri, C., Sinigaglia, M., 2005. Microbial characterization of table olives processed according to Spanish and natural styles. Food Technol. Biotechnol. 43, 289–294.

Chamkha, M., Sayadia, S., Brub, V., Godon, J.J., 2008. Microbial diversity in Tunisian olive fermentation brine as evaluated by small subunit rRNA e single strand conformation polymorphism analysis. Int. J. Food Microbiol. 122, 211–215.

Cocolin, L., Alessandria, V., Botta, C., Gorra, R., De Filippis, F., Ercolini, D., Rantsiou, K., 2013. NaOH-debittering induces changes in bacterial ecology during table olives fermentation. PLoS One 8 (7), e69074. http://dx.doi.org/10.1371/journal.pone.0069074.

Coton, E., Coton, M., Levert, D., Casaregola, S., Sohier, D., 2006. Yeast ecology in French cider and black olive natural fermentations. Int. J. Food Microbiol. 108, 130–135.

De Bellis, P., Valerio, F., Sisto, A., Lonigro, S.L., Lavermicocca, P., 2010. Probiotic table olives: microbial populations adhering on olive surface in fermentation sets inoculated with the probiotic strain *Lactobacillus paracasei* IMPC2.1 in an industrial plant. Int. J. Food Microbiol. 140, 6–13.

De Castro, A., Montaño, A., Casado, F.-J., Sánchez, A.-H., Rejano, L., 2002. Utilization of *Enterococcus casseliflavus* and *Lactobacillus pentosus* as starter culture for Spanish-style green olive fermentation. Food Microbiol. 19, 637–644.

Delgado, S., Arroyo, R., Martín, R., Rodríguez, J.M., 2008. PCR-DGGE assessment of the bacterial diversity of breast milk in women with lactational infectious mastitis. BMC Infect. Dis. 8, 51.

Doulgeraki, A.I., Hondrodimou, O., Eliopoulos, V., Panagou, E., 2012. Lactic acid bacteria and yeast heterogeneity during aerobic and modified atmosphere packaging storage of natural black Conservolea olives in polyethylene pouches. Food Control 26, 49–57.

Doulgeraki, A.I., Pramateftaki, P., Argyri, A.A., Nychas, G.J.E., Tassou, C.C., Panagou, E.Z., 2013. Molecular characterization of lactic acid bacteria isolated from industrially fermented Greek table olives. LWT Food Sci. Technol. 50, 353–356.

Ercolini, D., Villani, F., Aponte, M., Mauriello, G., 2006. Fluorescence in situ hybridisation detection of *Lactobacillus plantarum* group on olives to be used in natural fermentations. Int. J. Food Microbiol. 112, 291–296.

Fontana, L., Bermudez-Brito, M., Plaza-Díaz, J., Muñoz-Quezada, S., Gil, A., 2013. Sources, isolation, characterisation and evaluation of probiotics. Br. J. Nutr. 109, S35–S50.

Franzetti, L., Scarpellini, M., Vecchio, A., Planeta, D., 2011. Microbiological and safety evaluation of green table olives marketed in Italy. Ann. Microbiol. 61, 843–851.

Garrido Fernández, A., García García, P., Brenes Balbuena, M., 1995. Olive fermentations. In: Rehm, H.-J., Reed, G. (Eds.), Biotechnology: Enzymes, Biomass, Food and Feed. VCH, New York, pp. 593–627.

Garrido Fernández, A., Fernández Díez, M.J., Adams, R.M., 1997. In: Table Olives: Production and Processing. Chapman & Hall, London, pp. 134–197.

Golomb, B.L., Morales, V., Jung, A., Yau, B., Boundy-Mills, K.L., Marco, M.L., 2013. Effects of pectinolytic yeast on the microbial composition and spoilage of olive fermentations. Food Microbiol. 33, 97–106.

González Cancho, F., 1963. Microorganismos que se desarrollan en el aderezo de aceitunas verdes estilo español. Microbiol. Esp. 16, 221–230.

González-Cancho, F., 1965. Levaduras en la fermentación de aceitunas verdes estilo español. Estudio cuantitativo. Grasas Aceites 18, 230–234.

González Cancho, F., 1966a. Levaduras en la fermentación de aceitunas verdes estilo español (I). Rev. Cienc. Apl. 108, 24–31.

González Cancho, F., 1966b. Levaduras en la fermentación de aceitunas verdes estilo español (II). Rev. Cienc. Apl. 109, 125–131.

González-Cancho, F., Nosti-Vega, M., Durán-Quintana, M.C., Garrido-Fernández, A., Fernández-Diez, M.J., 1975. El proceso de fermentación en las aceitunas negras maduras en salmuera. Grasas Aceites 26, 297–309.

González-Cancho, F., Rejano, L., Borbolla y Alcalá, J.M.R., 1980. La formación de ácido propiónico durante la conservación de las aceitunas verdes de mesa. III. Microorganismos responsables. Grasas Aceites 31, 245–250.

González-Cancho, F., Durán-Quintana, M.C., 1981. Bacterias cocáceas del ácido láctico en el aderezo de aceitunas verdes. Grasas Aceites 32, 373–379.

Heperkan, D., 2013. Microbiota of table olive fermentations and criteria of selection for their use as starters. Front. Microbiol. 4, 143. http://dx.doi.org/10.3389/fmicb.2013.00143.

Hernández, A., Martin, A., Aranda, E., Pérez-Nevado, F., Córdoba, M.G., 2007. Identification and characterization of yeast isolated from the elaboration of seasoned green table olives. Food Microbiol. 24, 346–351.

Hurtado, A., Reguant, C., Esteve-Zarzoso, B., Bordons, A., Rozès, N., 2008. Microbial population dynamics during the processing of Arbequina table olives. Food Res. Int. 41, 738–744.

Hurtado, A., Reguant, A., Bordons, A., Rozès, N., 2012. Lactic acid bacteria from fermented table olives. Food Microbiol. 31, 1–8.

IOOC (International Olive Oil Council), 2012. Market Newsletter no. 66 – November. Spain, Madrid.

Kullen, M.J., Sanozky-Dawes, R.B., Crowell, D.C., Klaenhammer, T.R., 2000. Use of the DNA sequence of variable regions of the 16S rRNA gene for rapid and accurate identification of bacteria in the *Lactobacillus acidophilus* complex. J. Appl. Microbiol. 89, 511–516.

Kurtzman, C.P., Robnett, C.J., 1998. Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. Antonie van Leeuwenhoek 73, 331–371.

Lee, H.M., Lee, Y., 2008. A differential medium for lactic acid-producing bacteria in a mixed culture. Lett. Appl. Microbiol. 46, 676–681.

Legendre, P., Legendre, L., 1998. Ecological resemblance. In: Numerical EcologyDevelopments in Environmental Modelling, second ed., vol. 20. Elsevier, Amsterdam, pp. 247–302.

Magurran, A.E., 2004. How many species? In: Magurran, A.E. (Ed.), Measuring Biological Diversity. Blackwell, Oxford, pp. 72–130.

Maldonado-Barragán, A., Caballero-Guerrero, B., Lucena-Padrós, H., Ruiz-Barba, J.L., 2013. Induction of bacteriocin production by coculture is widespread among plantaricin-producing *Lactobacillus plantarum* strains with different regulatory operons. Food Microbiol. 33, 40–47.

Marquina, D., Peres, C., Caldas, F.V., Marques, F.V., Peinado, J.M., Spencer, M., 1992. Characterization of the yeast populations in olive brines. Lett. Appl. Microbiol. 14, 279–283.

Montaño, A., Sánchez, A.H., de Castro, A., 1993. Controlled fermentation of Spanish-style green olives. J. Food Sci. 58, 842–844.

Montaño, A., Sánchez, A.H., Casado, F.J., Beato, V.M., de Castro, A., 2013. Degradation of ascorbic acid and potassium sorbate by different *Lactobacillus* species isolated from packed green olives. Food Microbiol. 34, 7–11.

Mossel, D.A.A., Visser, M., Mengerink, W.H.J., 1962. A comparison of media for the enumeration of moulds and yeasts in foods and beverages. Lab. Pract. 11, 109–112.
Mourad, K., Nour-Eddine, K., 2006. Microbiological study of naturally fermented Algerian green olives: isolation and identification of lactic acid bacteria and yeasts along with the effects of brine solutions obtained at the end of olive fermentation on *Lactobacillus plantarum* growth. Grasas Aceites 57, 292–300.
Mrak, E.M., Vaughn, R.H., Millar, M.W., Phaff, H.J., 1956. Yeasts occurring in brines during the fermentation and storage of green olives. Food Technol. 10, 416–419.
Muccilli, S., Caggia, C., Randazzo, C.L., Restuccia, C., 2011. Yeast dynamics during the fermentation of brined green olives treated in the field with kaolin and Bordeaux mixture to control the olive fruit fly. Int. J. Food Microbiol. 148, 15–22.
Nisiotou, A.A., Chorianopoulus, N., Nychas, G.-J.E., Panagou, E.Z., 2010. Yeast heterogeneity during spontaneous fermentation of black Conservolea olives in different brine solutions. J. Appl. Microbiol. 108, 396–405.
Oliveira, M., Brito, D., Catulo, L., Leitao, F., Gomes, L., Silva, S., Vilas-Boas, L., Peito, A., Fernandes, I., Gordo, F., Peres, C., 2004. Biotechnology of olive fermentation of 'Galega' Portuguese variety. Grasas Aceites 55, 219–226.
Pereira, A.P., Pereira, J.A., Bento, A., Estevinho, M.L., 2008. Microbiological characterization of table olives commercialized in Portugal in respect to safety aspects. Food Chem. Toxicol. 46, 2895–2902.
Randazzo, C.L., Ribbera, A., Pitino, I., Romeo, F.V., Caggia, C., 2012. Diversity of bacterial population of table olives assessed by PCR-DGGE analysis. Food Microbiol. 32, 87–96.
Rejano, L., Montaño, A., Casado, F.J., Sánchez, A.H., de Castro, A., 2010. Table olives: varieties and variations. In: Preedy, V.R., Watson, R.R. (Eds.), Olives and Olive Oil in Health and Disease Prevention. Academic Press, Oxford, pp. 5–15.
Rodríguez-Gómez, F., Arroyo-López, F.N., López-López, A., Bautista-Gallego, J., Garrido-Fernández, A., 2010. Lipolytic activity of the yeast species associated with the fermentation/storage phase of ripe olive processing. Food Microbiol. 27, 604–612.
Ruiz-Barba, J.L., Piard, J.C., Jiménez-Díaz, R., 1991. Plasmid profiles and curing of plasmids in *Lactobacillus plantarum* strains isolated from green olive fermentation. J. Appl. Bacteriol. 71, 417–421.
Ruiz-Barba, J.L., Cathcart, D.P., Warner, P.J., Jiménez-Díaz, R., 1994. Use of *Lactobacillus plantarum* LPCO10, a bacteriocin producer, as a starter culture in Spanish-style green olive fermentation. Appl. Environ. Microbiol. 60, 2059–2064.
Ruiz-Barba, J.L., Jiménez-Díaz, R., 1995. Availability of essential B-group vitamins to *Lactobacillus plantarum* in green olive fermentation brines. Appl. Environ. Microbiol. 61, 1294–1297.
Ruiz-Barba, J.L., Maldonado, A., Jiménez-Díaz, R., 2005. Small-scale total DNA extraction from bacteria and yeast for PCR applications. Anal. Biochem. 347, 333–335.
Ruiz-Barba, J.L., Jiménez-Díaz, R., 2012. A novel *Lactobacillus pentosus*-paired starter culture for Spanish-style green olive fermentation. Food Microbiol. 30, 253–259.
Sánchez, A.H., Rejano, L., Montaño, A., de Castro, A., 2001. Utilization at high pH of starter cultures of lactobacilli for Spanish-style green olive fermentation. Int. J. Food Microbiol. 67, 115–122.
Sánchez, A.H., García, P., Rejano, L., 2006. Elaboration of table olives. Grasas Aceites 57, 86–94.
Silva, T., Reto, M., Sol, M., Peito, A., Peres, C.M., Peres, C., Xavier-Malcata, F., 2011. Characterization of yeasts from Portuguese brined olives, with a focus on their potentially probiotic behavior. LWT Food Sci. Technol. 44, 1349–1354.
Torriani, S., Felis, G.E., Dellaglio, F., 2001. Differentiation of *Lactobacillus plantarum, L. pentosus*, and *L. paraplantarum* by *recA* Gene Sequence Analysis and Multiplex PCR Assay with *recA* Gene-Derived Primers. Appl. Environ. Microbiol. 67, 3450–3454.
Ventura, M., Canchaya, C., Meylan, V., Klaenhammer, T.R., Zink, R., 2003. Analysis, characterization, and loci of the *tuf* genes in *Lactobacillus* and *Bifidobacterium* and their direct application for species identification. Appl. Environ. Microbiol. 69, 6908–6922.
Veyrat, A., Miralles, M.C., Pérez-Martínez, G., 1999. A fast method for monitoring the colonization rate of lactobacilli in a meat model system. J. Appl. Microbiol. 87, 49–61.
Whittaker, R.H., 1952. A study of summer foliage insect communities in the Great Smoky Mountains. Ecol. Monogr. 22, 1–44.
Zhou, J.Z., Jiang, Y., Deng, Y., Shi, Z., Zhou, B.Y., Xue, K., Wu, L., He, Z., Yang, Y., 2013. Random sampling process leads to overestimation of β-diversity of microbial communities. mBio 4 (3). http://dx.doi.org/10.1128/mBio.00324-13 e00324–13.

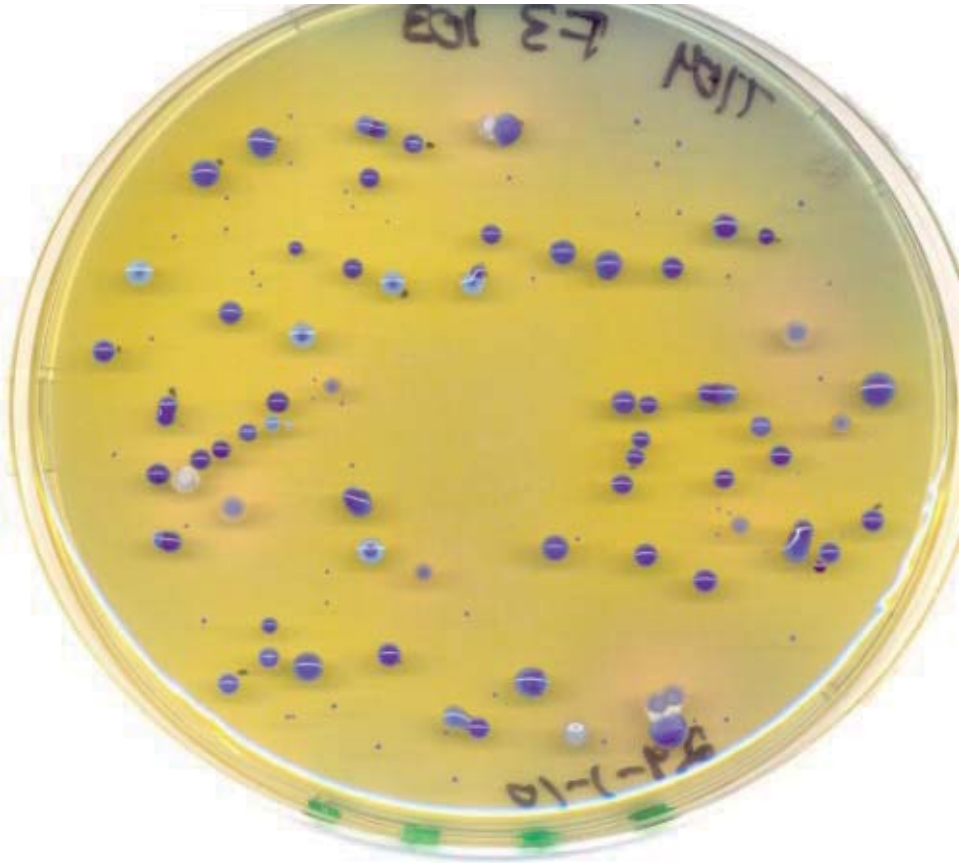

Figure S1. Colonies of bacteria growing onto MRS-BPB, a modified MRS-agar which included bromophenol blue (Lee and Lee, 2008) as a discriminating agent of the actual metabolism of the isolates.

4.2 *Enterococcus olivae* sp. nov., isolated from Spanish-style green olive fermentations.

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Enterococcus olivae sp. nov., isolated from Spanish-style green-olive fermentations

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Six strains of a hitherto unknown, Gram-stain-positive coccus were recovered from samples of Spanish-style green-olive fermentations. The 16S rRNA gene sequences from these isolates shared 98.7 % and 98.5 % of their nucleotide positions with those from *Enterococcus saccharolyticus* subsp. *taiwanensis* 812^T and from *E. saccharolyticus* subsp. *saccharolyticus* ATCC 43076^T, respectively. The sequence of the *rpoA* gene in the isolates was 95 % similar to that of *E. saccharolyticus* CECT 4309^T (=ATCC 43076^T). The 16S rRNA and *rpoA* gene phylogenies revealed that the isolates grouped in a statistically well-supported cluster separate from *E. saccharolyticus*. Enzyme activity profiles as well as fermentation patterns differentiated the novel bacteria from other members of the *Enterococcus* genus. Finally, phenotypic, genotypic and phylogenetic data supported the identification of a novel species of the genus *Enterococcus*, for which the name *Enterococcus olivae* sp. nov. is proposed. The type strain is IGG16.11^T (=CECT 8063^T=DSM 25431^T).

Enterococci are Gram-positive cocci belonging to the lactic acid bacteria (LAB) group and are ubiquitous in nature. They are commensal inhabitants of the gastrointestinal tract of mammalians and are also found in fermented foods, dairy products, plants, soil or water (Baele *et al.*, 2000; Devriese *et al.*, 1992; Devriese & Pot, 1995; Franz *et al.*, 1999; Klein, 2003). Enterococci can be involved in human nosocomial infections (Teixeira & Facklam, 2003), but they are also considered beneficial and safe in fermented products (Giraffa, 2002). At the time of this writing, according to the List of Prokaryotic names with Standing in Nomenclature (LPSN; http://www.bacterio.net), the genus *Enterococcus* consisted of 53 species and 2 subspecies.

Spanish-style green-olive fermentations appear to be dominated by *Lactobacillus pentosus* strains (Ruiz-Barba & Jiménez-Díaz, 2012), but enterococci seem to have a role at the crucial initial stage of these fermentations because of their tolerance to high pH and their LAB characteristics (De Castro *et al.*, 2002; Corsetti *et al.*, 2012). Enterococci isolated from olive fermentations mostly belong to the *Enterococcus casseliflavus* group (De Castro *et al.*, 2002; De Bellis *et al.*, 2010).

During the characterization of the microbiota associated to Spanish-style green-olive fermentations in two large table-olive manufacturing companies (4,000 to 8,000 tonnes of olives handled per season) in the province of Sevilla, south-western Spain, samples of the fermenting brines of ten 10-tonne fermenters were taken along the different fermentation stages at each fermentation yard (data not shown). These samples were analysed through culture-dependent techniques. The samples were serially diluted and spread on plates of de Man–Rogosa–Sharpe (MRS; Biokar Diagnostics) agar supplemented with 0.02 g bromophenol blue l^{-1} (AppliChem) and 0.05 % (w/v) L-cysteine (MRS-BPB; Lee & Lee, 2008), and on brain heart infusion (BHI; Biokar Diagnostics) agar supplemented with 0.05 % (w/v) L-cysteine. Plates were incubated anaerobically at 30 °C for 48 h. For anaerobic incubations, we used a DG250 Anaerobic Workstation (Don Whitley Scientific), with a gas mixture consisting of 10 % H_2, 10 % CO_2 and 80 % N_2. As a result, a total of 16 unidentified LAB isolates (fifteen from BHI and one from MRS-BPB) were detected. These isolates were present in all ten fermenters during the first fermentation stage (i.e. the first 10 days) at one of the

Abbreviations: LAB, lactic acid bacteria; RAPD, random amplified polymorphic DNA.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA sequences of *E. olivae* strains IGG16.11^T and IGG13.11 are JQ283454 and KJ566121, respectively. The accession numbers for the *rpoA* gene sequences of *E. olivae* strains IGG16.11^T and IGG13.11 are JQ283455 and KJ566122, respectively.

Two supplementary figures are available with the online version of this paper.

fermentation yards under study. The isolates were genotyped by RAPD by using the primer OPL5, following the protocol of Ruiz-Barba *et al.* (2005). The similarity of RAPD profiles was calculated using the pairwise Pearson's correlation coefficient, and the BioNumerics 6.6 (Applied Maths) software was used to reconstruct dendrograms using UPGMA analysis. Among the isolates, six different RAPD profiles were obtained (Fig. S1, available in the online Supplementary Material). One representative isolate of each RAPD profile was selected for further characterization. The study of the phenotypic and genotypic characteristics of these isolates indicated that they represented a novel species of the genus *Enterococcus*.

To determine the phylogenetic relationships of the isolates, the 16S rRNA gene was amplified with the primer pair 7 for (5′-AGAGTTTGATYMTGGCTCAG-3′) and 1510r (5′-TACGGYTACCTTGTTACGACTT-3′) (Lane, 1991), and both DNA strands were sequenced using the primers 7for, 785F (5′-GGATTAGATACCCBRGTAGTC-3′), mlb16 (5′-GGCTGCTGGCACGTAGTTAG-3′) (Kullen *et al.*, 2000) and 1510r. In addition, partial amplification and sequencing of 800 bp of the gene *rpoA* with primers rpoA-21-F (5′-ATGATYGARTTTGAAAAACC-3′) and rpoA-23-R (5′-ACHGTRTTRATDCCDGCRCG-3′) (Naser *et al.*, 2005) was performed.

The identification of the isolates and their phylogenetic neighbours was carried out using the 16S rRNA sequences obtained and the nucleotide BLAST (BLASTN) algorithm (Altschul *et al.*, 1997) to search a database on the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim *et al.*, 2012), containing type strains with updated, validly published prokaryotic names. The global alignment algorithm (Myers & Miller, 1988) available at EzTaxon-e was used to calculate pairwise sequence similarity. BLASTN (at http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare the DNA sequences of the *rpoA* gene in the isolates with those available in GenBank. DNA sequences belonging to type strains of the genus *Enterococcus* were obtained from the GenBank database (see Fig. 1). These sequences were aligned in CLUSTAL W (Thompson *et al.*, 1994) with MEGA 5 (version 5.2) software (Tamura *et al.*, 2007). Phylogenetic trees were reconstructed with the neighbour-joining method (Saitou and Nei, 1987). Bootstrapping analysis (1000 replicates) was used to evaluate the support of the groupings.

Chromosomal DNA was extracted following the method described by Cathcart (1995). The degree of DNA–DNA relatedness between strains IGG16.11^T and *Enterococcus saccharolyticus* CECT 4309^T was determined with a fluorometric method as described by Gonzalez & Saiz-Jimenez (2005). This method uses a real-time PCR thermocycler to measure the divergence between the thermal denaturation midpoint of homoduplex DNA and heteroduplex DNA (ΔT_m) via fluorescence values. In addition, the G+C content of genomic DNA of strain IGG16.11^T was determined by the fluorometric method described by Gonzalez & Saiz-Jimenez (2002). The results of both DNA–DNA relatedness and DNA G+C content were expressed as mean percentage and SD determined from three independent experiments.

The BLASTN analysis of the 16S rRNA gene sequences (1406 bp) from the six selected isolates indicated a similarity of 98.7 % with that of *E. saccharolyticus* subsp. *taiwanensis* 812^T and of 98.5 % with that of *E. saccharolyticus* subsp. *saccharolyticus* ATCC 43076^T. Similarly, BLAST analysis of the partial sequence (741 bp) of the *rpoA* gene from the isolates showed a similarity of 95.0 % with that of *E. saccharolyticus* CECT 4309^T (ATCC 43076^T). Both 16S rRNA and *rpoA* gene sequences were 100 % identical among the six isolates.

The phylogenetic analysis of the 16S rRNA sequence with the neighbour-joining method showed that the isolates IGG16.11^T and IGG13.11 formed a separate branch within the *Enterococcus gallinarum* group, with *E. saccharolyticus* ATCC 43076^T being the most closely related species (Fig. 1). Bootstrap resampling values of 70 % indicated statistically significant support for the *Enterococcus olivae* sp. nov. IGG16.11^T and IGG13.11 and *E. saccharolyticus* branch. The phylogenetic analysis of the *rpoA* gene sequence showed a clustering of *E. olivae* sp. nov. IGG16.11^T and IGG13.11 and *E. saccharolyticus*, with a bootstrap value of 99 % (Fig. S2). However, the topology of the tree was slightly different from that of the tree based on the 16S rRNA sequences.

The difference in melting temperature between genomic DNA from strain IGG16.11^T and from *E. saccharolyticus* CECT 4309^T was 7.5 °C. This value was above the 5 °C ΔT_m recommended as a cut-off point for the delineation of species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). DNA–DNA relatedness results confirmed that strain IGG16.11^T represents a novel species clearly differentiated from *E. saccharolyticus*. The G+C content of genomic DNA of strain IGG16.11^T was 40.4 mol% (SD, 0.1).

Substrate utilization, the fermentation/oxidation profile, acid production and some other physiological characteristics were examined using the API 50 CHL fermentation kit (bioMérieux) after 48 h of incubation according to the manufacturer's instructions. Tests for growth at various temperatures as well as in NaCl were performed in BHI broth as described by Švec *et al.* (2001) and Devriese *et al.* (1993). The results are given in the species description below. The six analysed strains revealed similar biochemical profiles enabling separation from other closely related enterococcal species. They could be differentiated from most known enterococcal species by their inability to produce acid from ribose, which is considered to be typical for the genus *Enterococcus*, although several of the more recently described species of the genus, e.g. *Enterococcus camelliae* and *Enterococcus italicus*, are also negative for this trait. Table 1 shows phenotypic tests that are useful for the differentiation of *E. olivae* sp. nov. IGG16.11^T from their closest phylogenetic relatives, *E. italicus*, *E. camelliae*, *E. saccharolyticus* and *Enterococcus sulfureus*.

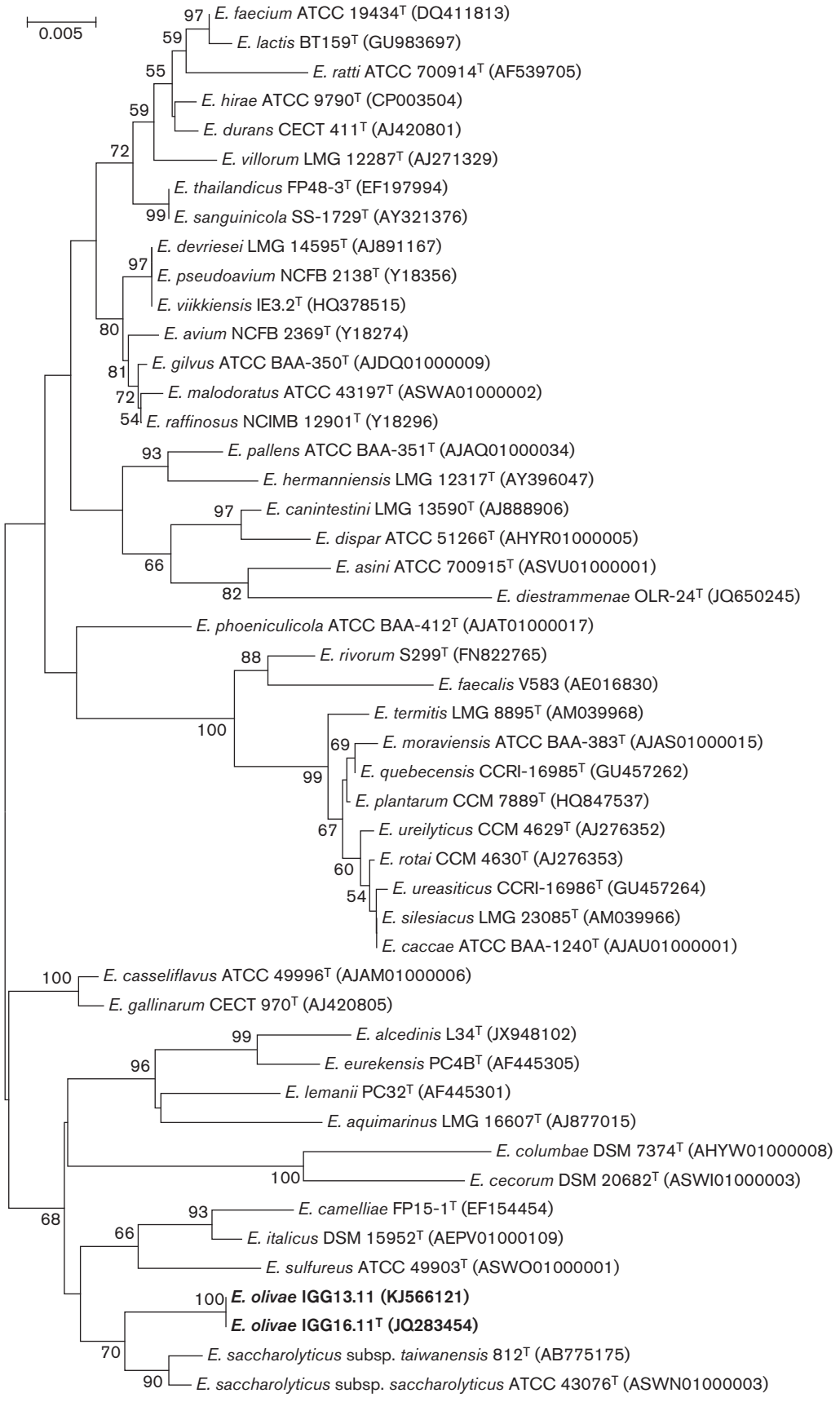
0.005
E. faecium ATCC 19434T (DQ411813)
E. lactis BT159T (GU983697)
E. ratti ATCC 700914T (AF539705)
E. hirae ATCC 9790T (CP003504)
E. durans CECT 411T (AJ420801)
E. villorum LMG 12287T (AJ271329)
E. thailandicus FP48-3T (EF197994)
E. sanguinicola SS-1729T (AY321376)
E. devriesei LMG 14595T (AJ891167)
E. pseudoavium NCFB 2138T (Y18356)
E. viikkiensis IE3.2T (HQ378515)
E. avium NCFB 2369T (Y18274)
E. gilvus ATCC BAA-350T (AJDQ01000009)
E. malodoratus ATCC 43197T (ASWA01000002)
E. raffinosus NCIMB 12901T (Y18296)
E. pallens ATCC BAA-351T (AJAQ01000034)
E. hermanniensis LMG 12317T (AY396047)
E. canintestini LMG 13590T (AJ888906)
E. dispar ATCC 51266T (AHYR01000005)
E. asini ATCC 700915T (ASVU01000001)
E. diestrammenae OLR-24T (JQ650245)
E. phoeniculicola ATCC BAA-412T (AJAT01000017)
E. rivorum S299T (FN822765)
E. faecalis V583 (AE016830)
E. termitis LMG 8895T (AM039968)
E. moraviensis ATCC BAA-383T (AJAS01000015)
E. quebecensis CCRI-16985T (GU457262)
E. plantarum CCM 7889T (HQ847537)
E. ureilyticus CCM 4629T (AJ276352)
E. rotai CCM 4630T (AJ276353)
E. ureasiticus CCRI-16986T (GU457264)
E. silesiacus LMG 23085T (AM039966)
E. caccae ATCC BAA-1240T (AJAU01000001)
E. casseliflavus ATCC 49996T (AJAM01000006)
E. gallinarum CECT 970T (AJ420805)
E. alcedinis L34T (JX948102)
E. eurekensis PC4BT (AF445305)
E. lemanii PC32T (AF445301)
E. aquimarinus LMG 16607T (AJ877015)
E. columbae DSM 7374T (AHYW01000008)
E. cecorum DSM 20682T (ASWI01000003)
E. camelliae FP15-1T (EF154454)
E. italicus DSM 15952T (AEPV01000109)
E. sulfureus ATCC 49903T (ASWO01000001)
E. olivae IGG13.11 (KJ566121)
E. olivae IGG16.11T (JQ283454)
E. saccharolyticus subsp. taiwanensis 812T (AB775175)
E. saccharolyticus subsp. saccharolyticus ATCC 43076T (ASWN01000003)

Fig. 1. Phylogenetic relationships based on comparison of 16S rRNA gene sequences (1406 nt) from strain IGG16.11^T with those from species of the genus *Enterococcus* described to date. For clarity, only isolates IGG16.11^T and IGG13.11 are shown in the tree. Nucleotide accession numbers of the sequences are given in parentheses. The tree was reconstructed with the neighbour-joining method. Numbers at branches indicate bootstrap values calculated for 1000 subsets for branch-points supported by values >50 %. Bar, 0.005 changes per nucleotide position.

On the basis of phenotypic, genotypic and phylogenetic characteristics, we suggest that the strains described here represent a novel species of the genus *Enterococcus*.

Description of *Enterococcus olivae* sp. nov.

Enterococcus olivae (o.li′vae. L. gen. n. *olivae* of an olive, referring to the isolation of the strains from olive fermentations).

Cells are Gram-stain-positive, non-motile, non-spore forming, egg shaped, 0.5–1 μm in diameter and arranged in pairs or in short chains. They are facultatively anaerobic, but grow better under anaerobic conditions on BHI plus 0.05 % (w/v) L-cysteine agar plates (BHI-Cys). Colonies grow on BHI-Cys after 2 days of incubation. Colonies are about 2–3 mm in diameter, circular, white, flat, with a smooth surface and entire edge, and exhibit the production of a 'ropy'-type exopolysaccharide. Colonies on Glucose Yeast-Extract Peptone medium (GYP) agar plates are circular, low-convex with entire margins, transparent and non-pigmented. Small, red colonies appear on Slanetz–Bartley agar. Positive for hydrolysis of aesculin and starch. Negative for catalase, hydrolysis of gelatin and production of gas from glucose and gluconate. Uses glucose fermentatively. No acidification, coagulation, reduction or liquefaction in skimmed milk after 7 days of growth. Growth in BHI at pH 5.0–9.6, with 0.5–6.5 % NaCl and at 10, 30 and 37 °C, but not at 45 °C. Acid is produced from D-glucose, D-fructose, D-galactose, D-mannose, maltose, aesculin, mannitol, *N*-acetylglucosamine, trehalose, sucrose, D-sorbitol, starch, melibiose, L-sorbose and raffinose. Weakly positive reaction in turanose and D-arabitol. Acid is not produced from glycerol, erythritol, D-/L-arabinose, D-ribose, D-/L-xylose, adonitol, methyl *β*-xyloside, L-rhamnose, dulcitol, inositol, D-amygdalin, inulin, melezitose, glycogen, xylitol, D-lyxose, D-/L-fucose, L-arabitol, gluconate or 2,5-diketogluconate.

The type strain, IGG16.11^T (=CECT 8063^T=DSM 25431^T), was isolated from Spanish-style green-olive fermentations. The DNA G+C content of the type strain is 40.4 mol%.

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Table 1. Biochemical characteristics that differentiate *E. olivae* sp. nov. from other related enterococci

Strains: 1, *E. olivae* sp. nov. IGG16.11^T; 2, E. camelliae FP15-1^T; 3, *E. italicus* DSM 15952^T; 4, *E. sulfureus* DSM 6905^T; 5, *E. saccharolyticus* subsp. *saccharolyticus* CECT 4309^T; 6, *E. saccharolyticus* subsp. *taiwanensis* 812^T. The biochemical characteristics of *E. saccharolyticus* subsp. *saccharolyticus* CECT 4309^T were experimentally determined in this study. Data for reference type strains were taken from Sukontasing *et al.* (2007), Fortina *et al.* (2004), Martinez-Murcia & Collins (1991), Farrow *et al.* (1984) and Chen *et al.* (2013). +, Positive; −, negative; W, weakly positive; ND, no data available.

Characteristic	1	2	3	4	5	6
Pigment production	−	−	−	+	−	−
Growth in 6.5 % NaCl	+	−	−	+	+	+
Growth at 10 °C	+	−	W	+	+	−
Production of acid from:						
D-Amygdalin	−	−	−	+	+	+
D-Arabitol	W	−	−	−	+	+
D-Galactose	+	−	+	+	+	W
Lactose	−	−	+	+	+	+
Melezitose	−	−	−	+	+	−
Melibiose	+	−	−	+	+	+
Raffinose	+	−	−	+	+	+
D-Ribose	−	−	−	+	+	−
D-Sorbitol	+	−	+	−	+	+
Turanose	W	−	−	ND	+	+
Potassium 2-ketogluconate	−	−	−	+	+	−
Starch	+	−	+	ND	+	−
DNA G+C content (mol%)	40.4	37.8	39.9–41.1	38.4	37.2	35.7

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References

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.

Baele, M., Baele, P., Vaneechoutte, M., Storms, V., Butaye, P., Devriese, L. A., Verschraegen, G., Gillis, M. & Haesebrouck, F. (2000). Application of tRNA intergenic spacer PCR for identification of *Enterococcus* species. *J Clin Microbiol* **38**, 4201–4207.

Cathcart, D. P. (1995). *Purification, characterization and molecular analysis of plantaricin S, a two-peptide bacteriocin from olive fermenting Lactobacillus plantarum strains*. PhD thesis, Cranfield University, Bedford, UK.

Chen, Y.-S., Lin, Y.-H., Pan, S.-F., Ji, S.-H., Chang, Y.-C., Yu, C.-R., Liou, M.-S., Wu, H.-C., Otoguro, M. & other authors (2013). *Enterococcus saccharolyticus* subsp. *taiwanensis* subsp. nov., isolated from broccoli. *Int J Syst Evol Microbiol* **63**, 4691–4697.

Corsetti, A., Perpetuini, G., Schirone, M., Tofalo, R. & Suzzi, G. (2012). Application of starter cultures to table olive fermentation: an overview on the experimental studies. *Front Microbiol* **3**, 248.

De Bellis, P., Valerio, F., Sisto, A., Lonigro, S. L. & Lavermicocca, P. (2010). Probiotic table olives: microbial populations adhering on olive surface in fermentation sets inoculated with the probiotic strain Lactobacillus paracasei IMPC2.1 in an industrial plant. *Int J Food Microbiol* **140**, 6–13.

de Castro, A., Montaño, A., Casado, F.-J., Sánchez, A.-H. & Rejano, L. (2002). Utilization of *Enterococcus casseliflavus* and *Lactobacillus pentosus* as starter culture for Spanish-style green olive fermentation. *Food Microbiol* **19**, 637–644.

Devriese, L. A. & Pot, B. (1995). The genus *Enterococcus*. In *The Genera of Lactic Acid Bacteria*, pp. 327–367. Edited by B. J. B. Wood & W. H. Holzapfel. London: Blackie Academic & Professional.

Devriese, L. A., Collins, M. D. & Wirth, R. (1992). The genus *Enterococcus*. In *The Prokaryotes: A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications*, pp. 1465–1481. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K. H. Schleifer. New York: Springer.

Devriese, L. A., Pot, B. & Collins, M. D. (1993). Phenotypic identification of the genus *Enterococcus* and differentiation of phylogenetically distinct enterococcal species and species groups. *J Appl Bacteriol* **75**, 399–408.

Farrow, J. A. E., Kruze, J., Phillips, B. A., Bramley, A. J. & Collins, M. D. (1984). Taxonomic studies on *Streptococcus bovis* and *Streptococcus equinus*: description of *Streptococcus alactolyticus* sp. nov. and *Streptococcus saccharolyticus* sp. nov. *Syst Appl Microbiol* **5**, 467–482.

Fortina, M. G., Ricci, G., Mora, D. & Manachini, P. L. (2004). Molecular analysis of artisanal Italian cheeses reveals *Enterococcus italicus* sp. nov. Int J Syst Evol Microbiol **54**, 1717–1721.

Franz, C. M. A. D., Holzapfel, W. H. & Stiles, M. E. (1999). Enterococci at the crossroads of food safety? *Int J Food Microbiol* **47**, 1–24.

Giraffa, G. (2002). Enterococci from foods. *FEMS Microbiol Rev* **26**, 163–171.

Gonzalez, J. M. & Saiz-Jimenez, C. (2002). A fluorimetric method for the estimation of G+C mol% content in microorganisms by thermal denaturation temperature. *Environ Microbiol* **4**, 770–773.

Gonzalez, J. M. & Saiz-Jimenez, C. (2005). A simple fluorimetric method for the estimation of DNA-DNA relatedness between closely related microorganisms by thermal denaturation temperatures. *Extremophiles* **9**, 75–79.

Kim, O. S., Cho, Y. J., Lee, K., Yoon, S. H., Kim, M., Na, H., Park, S. C., Jeon, Y. S., Lee, J. H. & other authors (2012). Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int J Syst Evol Microbiol* **62**, 716–721.

Klein, G. (2003). Taxonomy, ecology and antibiotic resistance of enterococci from food and the gastro-intestinal tract. *Int J Food Microbiol* **88**, 123–131.

Kullen, M. J., Sanozky-Dawes, R. B., Crowell, D. C. & Klaenhammer, T. R. (2000). Use of the DNA sequence of variable regions of the 16S rRNA gene for rapid and accurate identification of bacteria in the *Lactobacillus acidophilus* complex. *J Appl Microbiol* **89**, 511–516.

Lane, D. J. (1991). 16/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. Chichester: Wiley.

Lee, H. M. & Lee, Y. (2008). A differential medium for lactic acid-producing bacteria in a mixed culture. *Lett Appl Microbiol* **46**, 676–681.

Martinez-Murcia, A. J. & Collins, M. D. (1991). *Enterococcus sulfureus*, a new yellow-pigmented *Enterococcus* species. *FEMS Microbiol Lett* **80**, 69–74.

Myers, E. W. & Miller, W. (1988). Optimal alignments in linear space. *Comput Appl Biosci* **4**, 11–17.

Naser, S. M., Thompson, F. L., Hoste, B., Gevers, D., Dawyndt, P., Vancanneyt, M. & Swings, J. (2005). Application of multilocus sequence analysis (MLSA) for rapid identification of *Enterococcus* species based on *rpo*A and *phe*S genes. *Microbiology* **151**, 2141–2150.

Ruiz-Barba, J. L. & Jiménez-Díaz, R. (2012). A novel *Lactobacillus pentosus*-paired starter culture for Spanish-style green olive fermentation. *Food Microbiol* **30**, 253–259.

Ruiz-Barba, J. L., Maldonado, A. & Jiménez-Díaz, R. (2005). Small-scale total DNA extraction from bacteria and yeast for PCR applications. *Anal Biochem* **347**, 333–335.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Evol Microbiol* **44**, 846–849.

Sukontasing, S., Tanasupawat, S., Moonmangmee, S., Lee, J.-S. & Suzuki, K. (2007). *Enterococcus camelliae* sp. nov., isolated from fermented tea leaves in Thailand. *Int J Syst Evol Microbiol* **57**, 2151–2154.

Švec, P., Devriese, L. A., Sedlácek, I., Baele, M., Vancanneyt, M., Haesebrouck, F., Swings, J. & Doškař, J. (2001). *Enterococcus haemoperoxidus* sp. nov. and *Enterococcus moraviensis* sp. nov., isolated from water. *Int J Syst Evol Microbiol* **51**, 1567–1574.

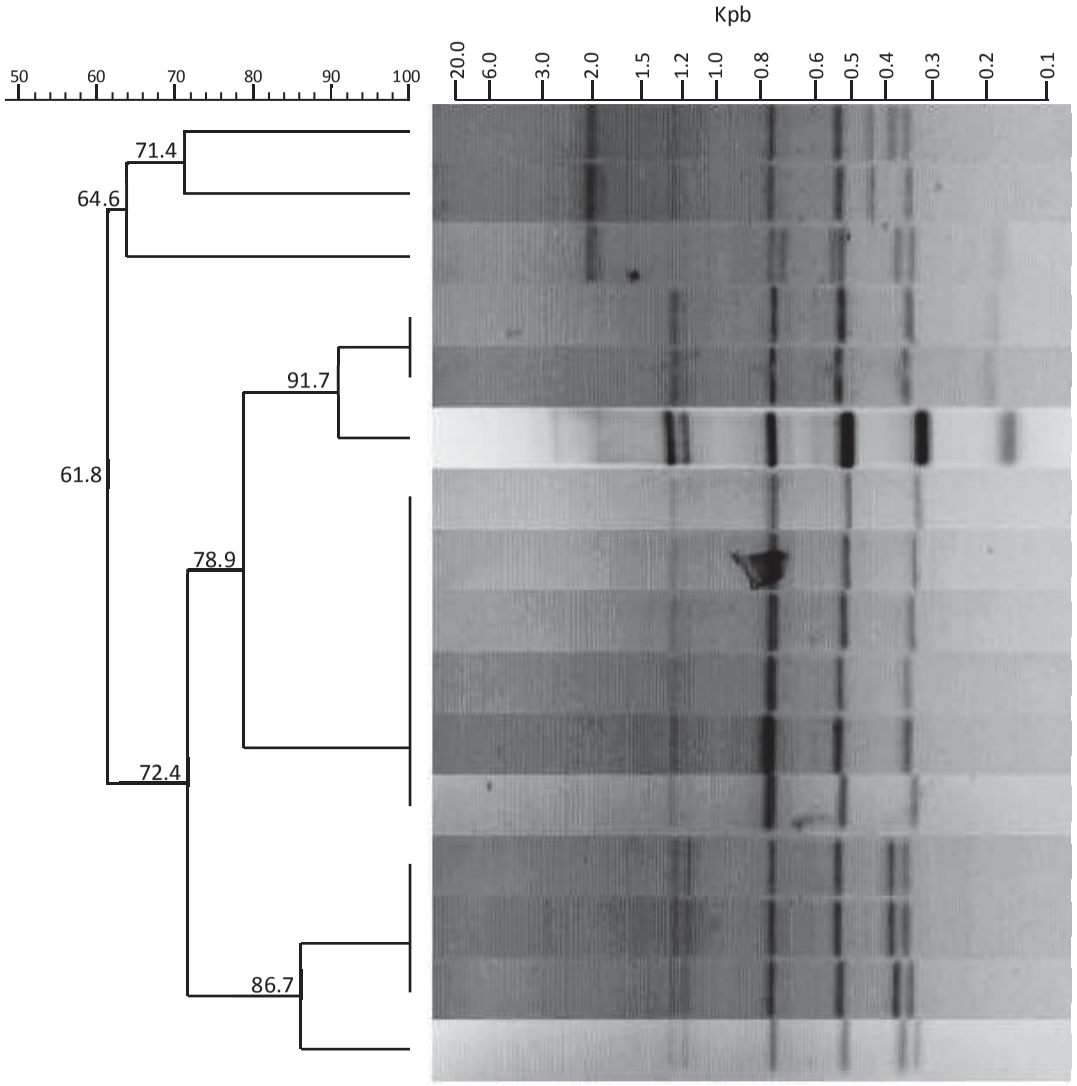
Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.

Teixeira, L. M. & Facklam, R. R. (2003). *Enterococcus*. In *Manual of Clinical Microbiology*, 8th edn, pp. 422–433. Edited by P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller & R. H. Yolken. Washington, DC: American Society for Microbiology.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

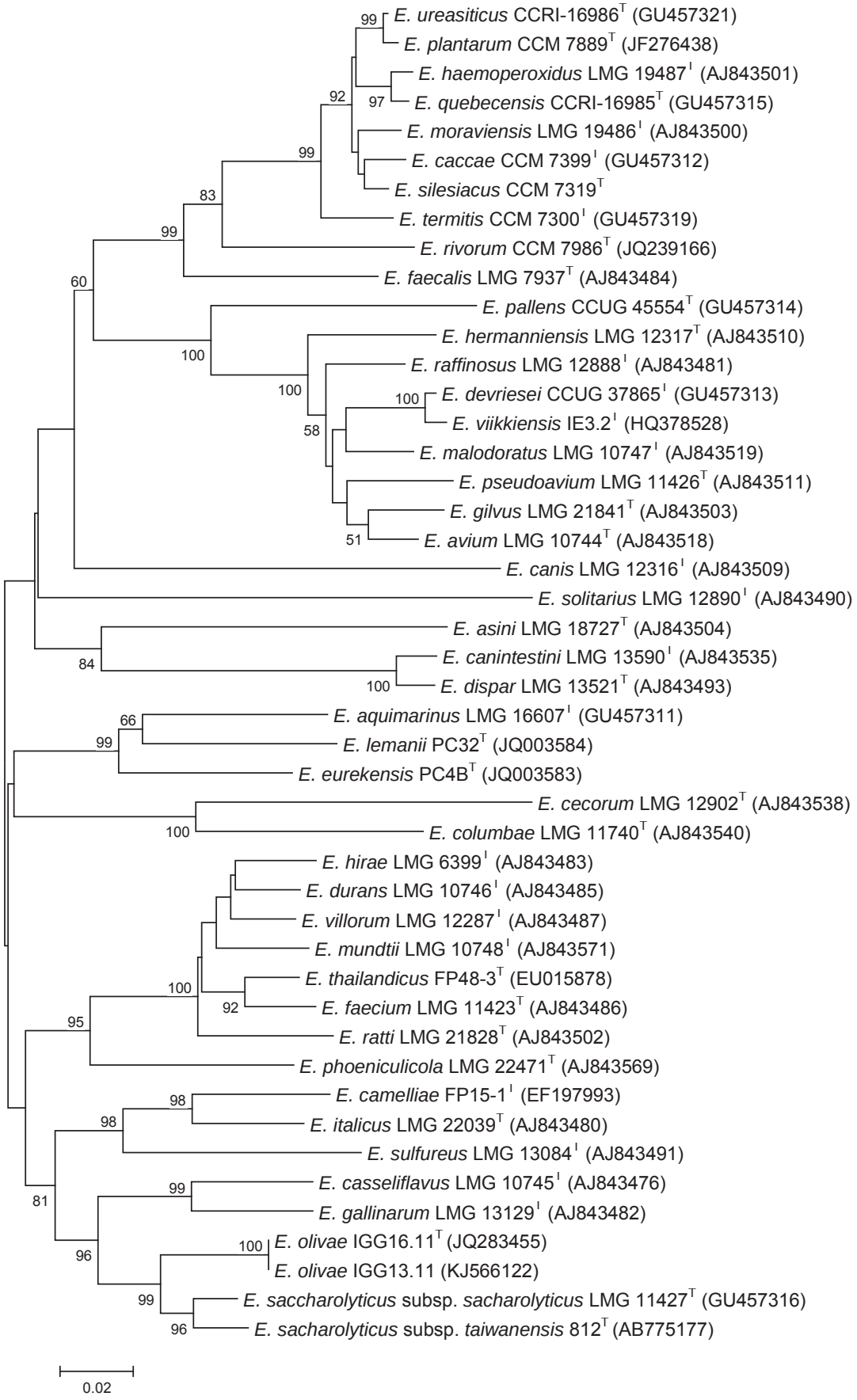
Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & other authors (1987). Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.

Figure S1. RAPD profiles and dendrogram (UPGMA) representing genetic relationships among *Enterococcus olivae* isolates based on genetic similarity matrix, calculated by using the pair-wise Pearson's correlation coefficient. The scale indicates the similarity level. The genotype indicates the grouping of the isolates according to their RAPD profile. The number of the fermenter where the isolated were recovered are indicated.



Isolate	Genotype	Fermenter
Enterococcus olivae IGG16.11^{T}	1	1
E. olivae IGG17.14	2	2
E. olivae IGG18.14	3	3
E. olivae IGG13.11	4	6
E. olivae IGG14.11	4	7
E. olivae IGG20.16	4	5
E. olivae IGG11.12	5	10
E. olivae IGG12.11	5	9
E. olivae IGG12.14	5	9
E. olivae IGG13.14	5	6
E. olivae IGG17.11	5	2
E. olivae IGG19.13	5	4
E. olivae IGG15.1	6	8
E. olivae IGG15.11	6	8
E. olivae IGG18.11	6	3
E. olivae IGG20.11	6	5

Figure S2. Phylogenetic relationships of *E. olivae* IGG16.11^T with most described *Enterococcus* species by comparison of *rpoA* gene sequences (741 nt). For clarity, only the isolates IGG16.11^T and IGG13.11 are shown. Nucleotide accession numbers for the sequences used are given in parentheses. The tree was based on the neighbour-joining method. Numbers on the tree indicate bootstrap values calculated for 1000 subsets for branch-points greater than 50%. Bar, 0.02 nucleotide changes per nucleotide position.



4.3 *Propionibacterium olivae* sp. nov. and *Propionibacterium damnosum* sp. nov., isolated from spoiled packaged Spanish-style green olives.

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Propionibacterium olivae sp. nov. and *Propionibacterium damnosum* sp. nov., isolated from spoiled packaged Spanish-style green olives

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Five strains of Gram-stain-positive bacteria were isolated from anomalous fermentations occurring in post-packaging of sealed airtight food-grade plastic pouches of Spanish-style green olives. These isolates could be grouped into two sets, which showed a similarity in their respective 16S rRNA gene sequences of 98.40 and 98.44 % with *Propionibacterium acidipropionici* NCFB 563 and 98.33 and 98.11 % with *Propionibacterium microaerophilum* M5^T, respectively, and a similarity of 99.41 % between them. The 16S rRNA gene phylogeny revealed that the isolates grouped into two statistically well-supported clusters separate from *P. acidipropionici* NCFB 563 and *P. microaerophilum* M5^T. Enzymic activity profiles as well as fermentation patterns differentiated these two novel bacteria from other members of the genus *Propionibacterium*. Finally, phenotypic, genotypic and phylogenetic data, supported the proposal of two novel species of the genus *Propionibacterium*, for which the names *Propionibacterium olivae* sp. nov. (type strain, IGBL1^T=CECT 8061^T=DSM 25436^T) and *Propionibacterium damnosum* sp. nov. (type strain, IGBL13^T=CECT 8062^T=DSM 25450^T) are proposed.

During the Spanish-style green-olive fermentations, there is an ecological succession of diverse micro-organism species. The first stage of the fermentation is characterized by the presence of *Enterobacteriaceae*, favoured by the high pH values reached after the alkaline treatment of the fruits that is characteristic of this olive preparation (Garrido-Fernández *et al.*, 1997; de Castro *et al.*, 2002). As the fermentation progresses, these micro-organisms disappear as consequence of the lowering of the pH due to the growth of lactic acid bacteria (LAB), mainly strains of *Lactobacillus pentosus*, which is characteristic of the second fermentation stage (de Castro *et al.*, 2002; Rejano *et al.*, 2010; Ruiz-Barba & Jiménez-Díaz, 2012). During the final, third stage of the fermentation, all fermentative substrates are exhausted and the LAB population declines steadily, thus starting the storage period. Values of pH below 4.0 and free acidities of 0.7–1.2 %, mainly as lactic acid, are considered indicative of a good fermentation so that these conditions, combined with NaCl concentrations usually raised to 7–8 %, should guarantee the long-term preservation of the final product. However, during the storage period, if brine conditioning is not appropriate, or after the packaging of the fruits, if the product is not pasteurized and/or the storage conditions are not appropriate, an undesirable secondary fermentation, usually led by species of the genus *Propionibacterium*, could occur (Sánchez *et al.*, 2006). These bacteria increase the pH because of the production of acetic and propionic acids from the lactic acid either produced during the previous phase of active fermentation or added at the time of packaging (Sánchez *et al.*, 2006). This implies a considerable microbiological risk because such changes may facilitate further growth of spoilage or even pathogen micro-organisms, such as those of the genus *Clostridium*, thus promoting different types of product spoilage (Kawatomari & Vaughn, 1956; Plastourgos & Vaughn, 1957; González Cancho *et al.*, 1973, 1980; Sánchez *et al.*, 2006). Three species of the genus *Propionibacterium*, namely '*Propionibacterium pentosaceum*', '*Propionibacterium zeae*' and *Propionibacterium acnes*, have been associated with 'zapatería' spoilage of olives (Plastourgos & Vaughn, 1957; González Cancho *et al.*, 1973, 1980). Propionibacteria are Gram-positive, catalase-positive, high G+C content, non-spore-forming

Abbreviations: LAB, lactic acid bacteria; RAPD, Random Amplified Polymorphic DNA.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence of *Propionibacterium olivae* IGBL1^T and *Propionibacterium damnosum* IGBL13^T are JQ283460 and JQ283461, respectively.

and non-motile bacteria, anaerobic or slightly aerotolerant and morphologically heterogeneous, that have a typical metabolism leading to the formation of propionic acid as the main end product of fermentation (Cummins & Johnson, 1986; Stackebrandt *et al.*, 2006). At the time of writing, the genus *Propionibacterium* consisted of 12 species and two subspecies (LPSN; http://www.bacterio.net/propionibacterium.html). Recently a novel species of the genus, '*Propionibacterium humerusii*' (Butler-Wu *et al.*, 2011), has been described, but it has not yet been validated.

The genus *Propionibacterium* has been traditionally divided into 'dairy' (*P. acidipropionici*, *P. cyclohexanicum*, *P. freudenreichii*, *P. jensenii*, *P. microaerophilum* and *P. thoenii*) and 'cutaneous' micro-organisms (*P. acidifaciens*, *P. acnes*, *P. australiense*, *P. avidum*, *P. granulosum* and *P. propionicum*) which mainly inhabit dairy/silage environments and the skin/intestine of human and animals, respectively. Dairy propionibacteria are generally recognized as safe micro-organisms being valuable for both technological applications and health promotion as probiotics, whereas members of the cutaneous group have shown to be opportunistic pathogens in compromised hosts (Cousin *et al.*, 2011).

Here we report the isolation of two novel species of the genus *Propionibacterium* from Spanish-style green-olives packaged in sealed airtight food-grade plastic pouches filled with covering liquid (NaCl, citric acid, lactic acid, L-ascorbic acid; pH 3.2) that had suffered spoilage after two-year storage at room temperature (packaging is usually for three years). Spoilage consisted of abnormal turbidity and unpleasant odour, as well as a non-appropriated, elevated pH value of 4.5. Plastic pouches, frequently used as packing material, cannot be pasteurized as heat accelerates polymerization of ortho-diphenols and a progressive browning of the product (Sánchez *et al.*, 1991).

Samples of the covering liquids from the plastic pouches were aseptically taken, serially diluted and spread onto agar plates of de Man-Rogosa-Sharpe (MRS; Biokar Diagnostics) supplemented with 0.02 g bromophenol blue l^{-1} (AppliChem) and 0.05 % (w/v) L-cysteine (MRS-BPB; Lee & Lee, 2008), brain heart infusion (BHI; Biokar Diagnostics) supplemented with 0.05 % (w/v) L-cysteine, Reinforced Clostridial Medium (RCM; Biokar Diagnostics), Yeast glucose LEMCO (Naylor & Sharpe, 1958) and MacConkey Broth Purple (Biokar Diagnostics). Plates were incubated anaerobically at 30 °C for 72 h. For anaerobic incubations, a DG250 Anaerobic Workstation (Don Whitley Scientific) was used, with a gas mixture consisting of 10 % H_2/10 % CO_2/80 % N_2.

Preliminary identification of the isolates was done by partial 16S rRNA gene sequence analysis (~450 bp). For this, PCR amplification with primers plb16 (5′-AGAGTTTGATCC-TGGCTCAG-3′) and mlb16 (5′-GGCTGCTGGCACGT-AGTTAG-3′) was carried out (Kullen *et al.*, 2000).

The isolates were genotyped by Random Amplified Polymorphic DNA (RAPD) profiling, following the protocol of Ruiz-Barba *et al.* (2005). *P. acidipropionici* NCFB 563 and *P. microaerophilum* DSM 13435^T (=M5^T) were also included in these analyses. The similarity of RAPD profiles was calculated using the Pearson's correlation coefficient, and Bionumerics 6.6 (Applied Maths) software was used to construct dendrograms based on UPGMA analysis.

To identify and determine the phylogenetic relatedness of the isolates, the 16S rRNA gene was amplified and sequenced (~1400 bp) with the primer pair 7for (5′-AGAGTTTGA-TYMTGGCTCAG-3′) and 1510r (5′-TACGGYTACCTTG-TTACGACTT-3′) (Lane, 1991). The identification of the isolates and their phylogenetic neighbours was carried out by the BLASTN program on the basis of 16S rRNA gene sequence data obtained (Altschul *et al.*, 1997) against the database containing type strains with updated validly published prokaryotic names, by using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim *et al.*, 2012). The calculation of pairwise sequence similarity was done using the global alignment algorithm (Myers & Miller, 1988), which was implemented at the EzTaxon-e server. The DNA sequences belonging to type strains of species of the genus *Propionibacterium* were obtained from the EzTaxon-e database. These sequences were aligned by using the CLUSTAL W method (Thompson *et al.*, 1994) with the MEGA 5 (version 5.2) software package (Tamura *et al.*, 2007). Phylogenetic trees were reconstructed based on the neighbour-joining method (Saitou & Nei, 1987). Bootstrapping analysis (1000 replicates) was done to study the stability of the groupings.

The degree of DNA–DNA relatedness between strain IGBL1^T, strain IGBL13^T, *P. microaerophilum* DSM 13435^T and *P. acidipropionici* NCFB 563 were determined by the fluorometric method as described by Gonzalez & Saiz-Jimenez (2005). This method measures the divergence between the thermal denaturation midpoint of homoduplex DNA and heteroduplex DNA (DTm) using a real-time PCR thermocycler that obtains fluorescence determinations. Chromosomal DNA was extracted following the method described by Cathcart (1995). The results were expressed as mean percentage values based on three independent hybridization experiments. The G+C content of genomic DNA of strains IGBL1^T and IGBL13^T was determined by the fluorometric method described by Gonzalez & Saiz-Jimenez (2002).

Substrate utilization, the fermentation/oxidation profile, acid production and some other physiological characteristics were examined using the API 50 CHL fermentation kit (bioMérieux) after 48 h of incubation according to the manufacturer's instructions. Cell morphology was examined by light microscopy (400×) using cells from exponentially growing cultures. Growth at different temperatures, pH and NaCl concentrations was determined on Yeast Glucose LEMCO anaerobically. Catalase activity was determined by bubble production in a 10 % (v/v) H_2O_2 solution. Metabolic end products (organic acids) were monitored through HPLC analysis of the cell-free supernatants of 48 h cultures in Yeast Glucose LEMCO broth incubated at 30 °C both aerobically (through shaking

at 150 r.p.m. in a Gallenkamp orbital incubator) and anaerobically. Growth in lactate was checked in Yeast Extract-Sodium Lactate medium, both aerobically and anaerobically.

Five isolates showing a high degree of similarity (99 %) in the partial 16S rRNA gene sequence with species of the genus *Propionibacterium* were isolated from RCM, LEMCO or BHI agar plates. Three of the isolates (IGBL1^T, IGBL13^T and IGB34) were isolated from the brine of the spoiled plastic pouch of production batch 'B' and the other two (IGB32 and IGBL3) from the spoiled plastic pouch of production batch 'BC'. Both batches had been elaborated on the same date.

The RAPD profile analysis (Fig. 1) showed three well-separated clusters, one of them corresponding to strains IGBL1^T, IGBL3 and IGBL34, a second one corresponding to strains IGBL13^T and IGBL32 and a third one including the reference strains *P. microaerophilum* DSM 13435^T and *P. acidipropionici* NCFB 563. One representative isolate from each RAPD group (IGBL1^T and IGBL13^T) was selected for further analysis.

The BLASTN analysis of strains IGBL1^T and IGBL13^T showed 16S rRNA gene sequence similarities of 98.40 and 98.44 %, respectively, with *P. acidipropionici* NCFB 563, and 98.33 and 98.11 %, respectively, with *P. microaerophilum* M5^T, and a similarity of 99.41 % between the novel strains.

The phylogenetic analysis inferred from the 16S rRNA gene sequences using the neighbour-joining method, showed that the isolates IGBL1^T, IGBL3 and IGBL34, and IGBL13^T and IGBL32 formed two new sub-lines within the genus *Propionibacterium*. Bootstrap resampling values (94 %) showed that branches of strains IGBL1^T and IGBL13^T were statistically significant (Fig. 2).

The difference in melting temperature between genomic DNA from strain IGBL1^T and that from strain IGBL13^T, *P. microaerophilum* DSM 13435^T and *P. acidipropionici* NCFB 563 were 9.0, 5.2 and 10.4 °C, respectively. In the same manner, the difference in melting temperature between genomic DNA from strain IGBL13^T and that from strain IGBL1^T, *P. microaerophilum* DSM 13435^T and *P. acidipropionici* NCFB 563 were 9.0, 7.2 and 7.0 °C. These values were all above the 5 °C ΔTm recommended as cut-off points for the delineation of species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). DNA–DNA relatedness results confirmed that both strains IGBL1^T and IGBL13^T represent two novel species clearly differentiated from *P. microaerophilum* DSM 13435^T and *P. acidipropionici* NCFB 563. The G+C content of genomic DNA of strains IGBL1^T and IGBL13^T were 70.0 mol% (SD 1.0) and 67.5 mol% (SD 0.2), respectively. This range is in agreement with that described for other species of the genus *Propionibacterium* (Table 1).

The three analysed strains of the first novel species (IGBL1^T, IGBL3 and IGBL34) were shown to possess the same biochemical profile described in Table 1 for the type strain. In the same manner, the two strains of the second novel species (IGBL13^T and IGBL32) showed the same biochemical profile. Despite the high similarity at their 16S rRNA gene sequences, both novel species can be easily differentiated attending to their biochemical characteristics. Table 1 shows phenotypical tests that are useful for differentiation between the two novel species and from their closest phylogenetic relatives, i.e. *P. microaerophilum*

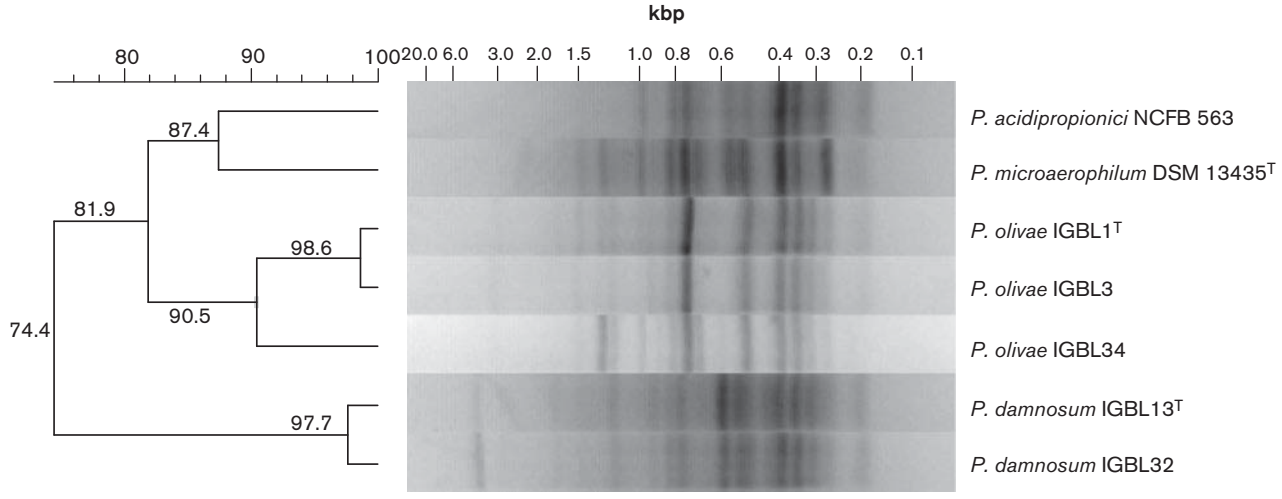

Fig. 1. RAPD profiles and dendrogram (UPGMA) representing genetic relationships among isolates of *Propionibacterium olivae* sp. nov. and *Propionibacterium damnosum* sp. nov. and the reference strains *P. acidipropionici* NCFB 563 and *P. microaerophilum* DSM 13435^T (=M5^T) based on genetic similarity matrix, calculated by using the pair-wise Pearson's correlation coefficient. Bar, similarity level (%).

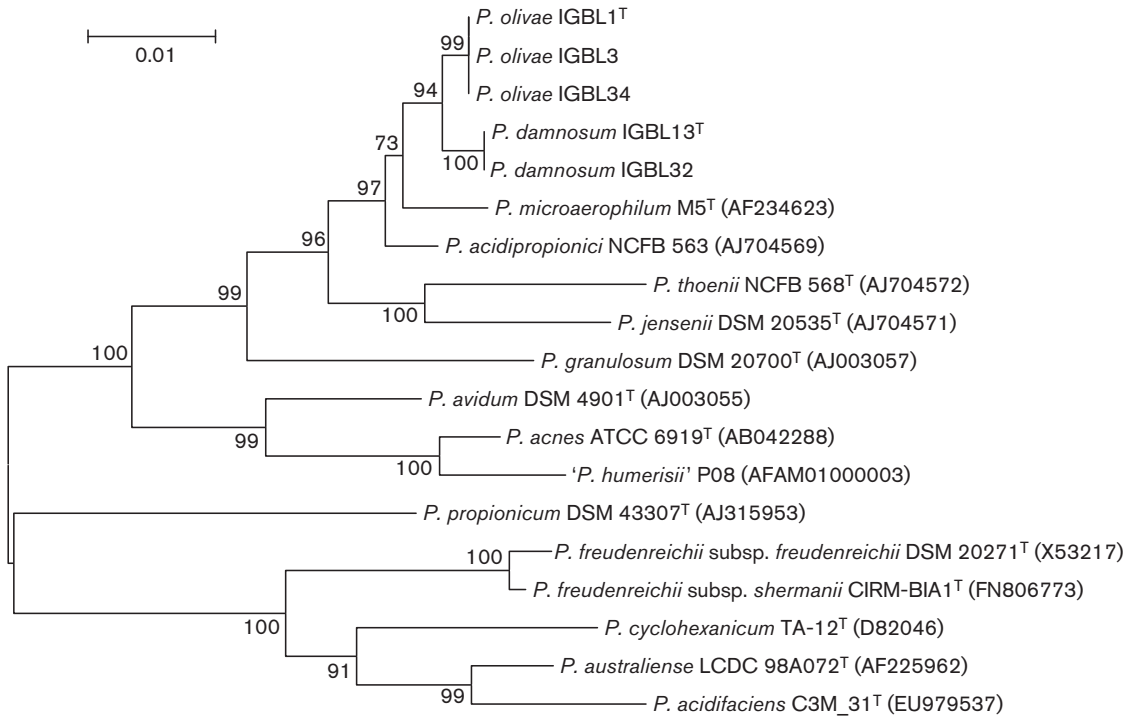

Fig. 2. Phylogenetic relationships of *P. olivae* sp. nov. IGBL1^T, IGBL3 and IGB34, and *P. damnosum* sp. nov. IGBL13^T and IGB32 with strains of species of the genus *Propionibacterium* based on comparison of 16S rRNA gene sequences (1406 nt). GenBank accession numbers for the sequences used are given in parentheses. The tree was based on the neighbour-joining method. Numbers on the tree indicate bootstrap values calculated for 1000 subsets for branch-points greater than 50 %. Bar, 0.01 nt changes per nucleotide position.

M5^T, *P. acidipropionici* NCFB 563, *P. jensenii* DSM 20535^T and *P. thoenii* DSM 20276^T.

Strain IGBL1^T grows at a pH range of 4.0 to 10.0, 4.0 % concentration of NaCl, in aerobic conditions at 30 °C, in the same manner as *P. microaerophilum* DSM 13435^T and *P. acidipropionici* NCFB 563. Strain IGBL13^T, however, shows weak growth at pH 4, 4.0 % NaCl, and aerobic conditions at 30 °C, showing no growth at pH 9.0 or pH 10.0. Interestingly, *P. microaerophilum* DSM 13435^T, one of the closest taxa to these novel species was originally isolated from a similar environmental niche (olive mill wastewater; Koussémon *et al.*, 2001).

Cell morphology under the microscope was especially characteristic in strain IGBL13^T, which was able to form filaments, sometimes branched, of various lengths (5–30 μm). This characteristic has been previously observed in *P. propionicum* and strains of *P. acnes* phylotype III.

In conclusion, on the basis of DNA–DNA reassociation values and phenotypic, genotypic and phylogenetic characteristics, we suggest the existence of two novel species of the genus *Propionibacterium*, for which the names *Propionibacterium olivae* sp. nov. (type strain IGBL1^T) and *Propionibacterium damnosum* sp. nov. (type strain IGBL13^T) are proposed.

Description of *Propionibacterium olivae* sp. nov.

Propionibacterium olivae (o.li′vae L. gen. n. *olivae* of an olive, referring to the isolation of the strains from olives).

Cells are Gram-positive, facultatively anaerobic, aerotolerant, catalase-positive, non-motile, non-spore-forming, pleomorphic bacilli with rounded edges (0.7–0.8 × 1.4–4.0 μm) and arranged in pairs or in short chains in aged cultures (Fig. 3a). Colonies on Yeast Glucose LEMCO agar are circular with entire margins, highly convex, white to cream, opaque, creamy, and 3 mm in diameter. Growth in broth media produces a homogeneous turbidity. Positive for hydrolysis of aesculin but negative for starch. Cells ferment glycerol, erythritol, D- and L-arabinose, D-ribose, D-xylose, adonitol, D-glucose, D-galactose, D-fructose, D-mannose, inositol, mannitol, D-sorbitol, arbutin, salicin, maltose, cellobiose, trehalose, sucrose, xylitol, turanose, D- and L-arabitol. Grows in lactate, both aerobically and anaerobically. Does not ferment L-xylose, methyl β-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, methyl α- D-mannopyranoside, methyl α-D-glucopyranoside, *N*-acetylglucosamine, D-amygdalin, lactose, melibiose, inulin, melezitose, raffinose, glycogen, gentiobiose, D-lyxose, D-tagatose, D- or L-fucose, gluconate or 2- or 5-ketogluconate. Grows at a temperature range of 20 to 42 °C (with optimal growth at 30 °C), at a pH range of 4.0 to 10.0 and

Table 1. Biochemical characteristics that differentiate *Propionibacterium olivae* sp. nov. and *Propionibacterium damnosum* sp. nov. from other related propionibacteria

Strains: 1, *P. olivae* sp. nov. IGBL1^T; 2, *P. damnosum* sp. nov. IGBL13^T; 3, *P. microaerophilum* DSM 13435^T (=M5^T); 4, *P. acidipropionici* NCFB 563; 5, *P. jensenii* DSM 20535^T; 6, *P. thoenii* DSM 20276^T. The biochemical characteristics of taxa 1 and 2 were determined in this study, while those of taxa 3–6 were taken from Koussémon *et al.* (2001) and Kusano *et al.* (1997). +, Positive; –, negative; +/–, result depending on the authors; ND, no data available.

Characteristic	1	2	3	4	5	6
Production of acid from:						
D-Arabinose	+	–	–	+	–	–
L-Arabinose	+	+	+	+	–	–
D-Xylose	+	–	–	+	–	–
L-Sorbose	–	–	+	–	–	–
Rhamnose	–	+	+	+	–	–
N-Acetylglucosamine	–	+	+	+	–	+
Amygdalin	–	–	–	+/–	+	–
Arbutin	+	–	+	+	ND	ND
Cellobiose	+	+	+	+	ND	ND
Lactose	–	+	–	+	–	+
Melibiose	–	+	–	+	+	–
Melezitose	–	–	+	+	+	–
Raffinose	–	–	–	+/–	+	–
Starch	–	–	+	+	–	+
Glycogen	–	–	+	+/–	–	–
Gluconate	–	–	+	+	+	–
DNA G+C content (mol%)	70.0	67.5	67.1	67.5	66	66

in the presence of 4.0 % NaCl. No growth was observed after pasteurization at 80 °C for 10 min. Under anaerobic conditions, propionic and acetic acids are formed from glucose fermentation, while only acetic acid, plus a small amount of succinic acid are formed when grown aerobically.

The type strain, IGBL1^T (=CECT 8061^T=DSM 25436^T), was isolated from the brines of plastic pouches of packaged, non-pasteurized, two-year-stored Spanish-style green olives that suffered spoilage consisting of abnormal turbidity and unpleasant odour. The DNA G+C content of the type strain is 70.0 mol%.

Description of *Propionibacterium damnosum* sp. nov.

Propionibacterium damnosum (dam.no′sum. L. neut. adj. *damnosum* causing losses, referring to the isolation of the type strain from spoiled olives).

Cells are Gram-positive, facultatively anaerobic, aerotolerant, catalase-negative, non-motile, non-spore-forming, pleomorphic rods and are able to form filaments, sometimes branched, variable in length (0.6–0.7 × 5–30 μm) (Fig. 3b). Colonies on LEMCO agar are circular with irregular margins, white to cream, opaque, creamy, rough and 2–3 mm in diameter. In broth culture, the cells form aggregates having a granular appearance and being deposited at the bottom of the tubes. Hydrolyses aesculin but not starch. Cells ferment glycerol, erythritol, L-arabinose, D-ribose, adonitol, D-glucose, D-galactose, D-fructose, D-mannose, L-rhamnose, inositol, mannitol, D-sorbitol, *N*-acetylglucosamine, cellobiose, lactose, melibiose, sucrose, trehalose, xylitol, turanose and D- and L-arabitol. Grows in lactate, both aerobically and anaerobically. Does not ferment D-arabinose, D- or L-xylose, methyl *β*-D-xylopyranoside, L-sorbose, dulcitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, D-amygdalin, arbutin, salicin, maltose, inulin, melezitose, raffinose, glycogen, gentiobiose, D-lyxose, D-tagatose, D- or L-fucose, gluconate or 2- or 5-ketogluconate. Broth cultures grow well at a temperature range of 20 to 42 °C (with optimal growth at 30 °C), at a pH range of 4.5 to 8 and in the presence of 2.0 % NaCl, showing weak growth with 4.0 % NaCl. No growth was observed after pasteurization at 80 °C for 10 min. Under anaerobic conditions, propionic and acetic acids are formed from glucose fermentation, while only acetic acid is formed when grown aerobically.

The type strain, IGBL13^T (=CECT 8062^T=DSM 25450^T), was isolated from the brines of plastic pouches of packaged, non-pasteurized, two-year-stored Spanish-style green olives that suffered spoilage consisting of abnormal turbidity and unpleasant odour. The DNA G+C content of the type strain is 67.5 mol%.

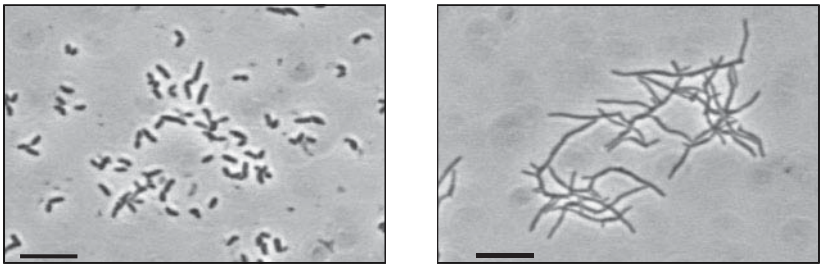

Fig. 3. Phase-contrast photomicrograph of *Propionibacterium olivae* sp. nov. IGBL1^T (a) and *Propionibacterium damnosum* sp. nov. IGBL13^T (b) from cultures in the stationary phase of growth. Bars, 10 μm.

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References

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.

Butler-Wu, S. M., Sengupta, D. J., Kittichotirat, W., Matsen, F. A., III & Bumgarner, R. E. (2011). Genome sequence of a novel species, *Propionibacterium humerusii*. *J Bacteriol* **193**, 3678.

Cathcart, D. P. (1995). Purification, characterization and molecular analysis of plantaricin S, a two-peptide bacteriocin from olive fermenting *Lactobacillus plantarum* strains. Ph.D. Thesis. Cranfield University, Bedford, United Kingdom.

Cousin, F. J., Mater, D. D. G., Foligné, B. & Jan, G. (2011). Dairy propionibacteria as human probiotics: a review of recent evidence. *Dairy Sci Technol* **91**, 1–26.

Cummins, C. S. & Johnson, J. L. (1986). Genus I. *Propionibacterium*. In: *Bergey's Manual of Systematic Bacteriology*, 1st edn, pp. 1346–1353. Edited by J. G. Holt. Baltimore: Williams & Wilkins.

de Castro, A., Montaño, A., Casado, F.-J., Sánchez, A.-H. & Rejano, L. (2002). Utilization of *Enterococcus casseliflavus* and *Lactobacillus pentosus* as starter culture for Spanish-style green olive fermentation. *Food Microbiol* **19**, 637–644.

Garrido Fernández, A., Fernández Díez, M. J. & Adams, M. R. (1997). Green olive type. In *Table Olives: Production and Processing*, pp. 134–206. Edited by A. Garrido Fernández, M. J. Fernández Díez & M. R. Adams. London: Chapman & Hall.

Gonzalez, J. M. & Saiz-Jimenez, C. (2002). A fluorimetric method for the estimation of G+C mol% content in microorganisms by thermal denaturation temperature. *Environ Microbiol* **4**, 770–773.

Gonzalez, J. M. & Saiz-Jimenez, C. (2005). A simple fluorimetric method for the estimation of DNA-DNA relatedness between closely related microorganisms by thermal denaturation temperatures. *Extremophiles* **9**, 75–79.

González Cancho, F., Fernández Díez, M. J., Nosti Vega, M. & Buzcu, N. (1973). Caracteristiques des principaux micro-organismes responsables de la ''zapateria''. *Inf Oleicoles Int* **60–61**, 133–144.

González Cancho, F., Rejano Navarro, L. & Borbolla y Alcalá, J. M. R. (1980). La formacion de ácido propiónico durante la conservación de aceituna verdes de mesa. III. Micro-organismos responsables. *Grasas Aceites* **31**, 245–250.

Kawatomari, T. & Vaughn, R. H. (1956). Species of *Clostridium* associated with zapatera spoilage of olives. *J Food Sci* **21**, 481–490.

Kim, O. S., Cho, Y. J., Lee, K., Yoon, S. H., Kim, M., Na, H., Park, S. C., Jeon, Y. S., Lee, J. H. & other authors (2012). Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int J Syst Evol Microbiol* **62**, 716–721.

Koussémon, M., Combet-Blanc, Y., Patel, B. K., Cayol, J. L., Thomas, P., Garcia, J. L. & Ollivier, B. (2001). *Propionibacterium microaerophilum* sp. nov., a microaerophilic bacterium isolated from olive mill wastewater. *Int J Syst Evol Microbiol* **51**, 1373–1382.

Kullen, M. J., Sanozky-Dawes, R. B., Crowell, D. C. & Klaenhammer, T. R. (2000). Use of the DNA sequence of variable regions of the 16S rRNA gene for rapid and accurate identification of bacteria in the *Lactobacillus acidophilus* complex. *J Appl Microbiol* **89**, 511–516.

Kusano, K., Yamada, H., Niwa, M. & Yamasato, K. (1997). *Propionibacterium cyclohexanicum* sp. nov., a new acid-tolerant ω-cyclohexyl fatty acid-containing propionibacterium isolated from spoiled orange juice. *Int J Syst Bacteriol* **47**, 825–831.

Lane, D. J. (1991). 16/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. Chichester: Wiley.

Lee, H. M. & Lee, Y. (2008). A differential medium for lactic acid-producing bacteria in a mixed culture. *Lett Appl Microbiol* **46**, 676–681.

Myers, E. W. & Miller, W. (1988). Optimal alignments in linear space. *Comput Appl Biosci* **4**, 11–17.

Naylor, J. & Sharpe, M. E. (1958). Lactobacilli in cheddar cheese: 1. The use of selective media for isolation and of serological typing for identification. *J Dairy Res* **25**, 92–103.

Plastourgos, S. & Vaughn, R. H. (1957). Species of *Propionibacterium* associated with zapatera spoilage of olives. *Appl Microbiol* **5**, 267–271.

Rejano, L., Montaño, A., Casado, F. J., Sánchez, A. H. & de Castro, A. (2010). Table olives: varieties and variations. In Olives and Olive Oil in Health and Disease Prevention, pp. 5–15. Edited by V. R. Preedy & R. R. Watson. Oxford: Academic Press.

Ruiz-Barba, J. L. & Jiménez-Díaz, R. (2012). A novel *Lactobacillus pentosus*-paired starter culture for Spanish-style green olive fermentation. *Food Microbiol* **30**, 253–259.

Ruiz-Barba, J. L., Maldonado, A. & Jiménez-Díaz, R. (2005). Small-scale total DNA extraction from bacteria and yeast for PCR applications. *Anal Biochem* **347**, 333–335.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Sánchez, A. H., Rejano, L. & Montaño, A. (1991). Kinetics of the destruction by heat of colour and texture of pickled green olives. *J Sci Food Agric* **54**, 379–385.

Sánchez Gomez, A. H., García, P. G. & Rejano Navarro, L. (2006). Elaboration of table olives. *Grasas Aceites* **57**, 86–94.

Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.

Stackebrandt, E., Cummins, C. S. & Johnson, J. L. (2006). Family *Propionibacteriaceae*: the genus *Propionibacterium*. In *The Prokaryotes: A Handbook on the Biology of Bacteria*, pp. 400–418. Edited by M. Dworkin, S. Falkow, E. Rosenberg, K. H. Schleifer & E. Stackebrandt. New York: Springer.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.

4.4 Genetic diversity and dynamics of bacterial and yeast strains associated to Spanish-style green table-olive fermentations in large manufacturing companies.

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Genetic diversity and dynamics of bacterial and yeast strains associated to Spanish-style green table-olive fermentations in large manufacturing companies

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ABSTRACT

We have genotyped a total of 1045 microbial isolates obtained along the fermentation time of Spanish-style green table olives from the fermentation yards (*patios*) of two large manufacturing companies in the Province of Sevilla, south of Spain. Genotyping was carried out using RAPD-PCR fingerprinting. In general, isolates clustered well into the relevant phylogenetic dendrograms, forming separate groups in accordance to their species adscription. We could identify which bacterial and yeast genotypes (strains) persisted throughout the fermentation at each *patio*. Also, which of them were more adapted to any of the three stages, i.e. initial, middle and final, described for this food fermentation. A number of genotypes were found to be shared by both *patios*. Fifty seven of these belonged to five different bacterial species, i.e. *Lactobacillus pentosus*, *Lactobacillus paracollinoides/collinoides*, *Lactobacillus rapi*, *Pediococcus ethanolidurans* and *Staphylococcus* sp., although most of them (51) belonged to *L. pentosus*. Four yeast genotypes were also shared, belonging to the species *Candida thaimueangensis*, *Saccharomyces cerevisiae* and *Hanseniaspora* sp. Two genotypes of *L. pentosus* were found to be grouped with those of two strains used in commercially available starter cultures, one of them bacteriocinogenic, which were used up to three years before this study in these *patios*, demonstrating the persistence of selected strains in this environment. Biodiversity was assessed though different indexes, including richness, diversity and dominance. A statistically significant decrease in biodiversity between the initial and final stages of the fermentation was found in both *patios*. However, values of biodiversity indexes in the fermenters were very similar, and no significant differences were found in the total biodiversity between both *patios*. This study allowed us to identify a range of well adapted strains (genotypes), especially those belonging to the lactic acid bacteria, which could be useful to improve safety and quality of table olive fermentations.

1. Introduction

Production of table olives is of major economical and social importance in Mediterranean countries (Garrido Fernández et al., 1997). This vegetable fermentation can be produced through a wide variety of methods, although most of the world production is carried out by one of the three most common industrial preparations, i.e. Spanish-style, natural black olives and oxidized black olives (Rejano et al., 2010). Spanish-style fermentation of green olives represents up to 60% of the world table olive production (Botta and Cocolin, 2012), most of it being concentrated in Spain. This table olive preparation is characterized by an initial alkali (NaOH) treatment of the green fruits to remove most of their natural bitterness as well as bacterial-growth inhibitory compounds, mostly phenolic compounds (Rejano et al., 2010). A subsequent spontaneous fermentation takes usually place in the brine which finally covers the alkali-treated olives. This fermentation is mainly carried out by lactic acid bacteria (LAB) and, more precisely, strains of the species *Lactobacillus pentosus* (de Castro et al., 2002; Rejano et al., 2010; Ruiz-Barba and Jiménez-Díaz, 2012).

In a recent publication (Lucena-Padrós et al., 2014), we reported a comprehensive study on the microbiota, bacterial and yeast species, that is associated to the Spanish-style fermentation of green olives in large-scale table olive manufacturing companies located in the Province of Sevilla, southern Spain, where actually up to 63% of the national production is manufactured (season 2012/2013; AAO, 2013). In that study, more than one thousand isolates were obtained and identified at the species level using molecular criteria. In particular, 37 different bacterial species were isolated, belonging to 18 different genera, while 12 yeast species were isolated, belonging to 7 distinct genera. Moreover, a substantial number of bacterial and yeast species had not been described before either from Spanish-style olive fermentations or even from table olives, including a novel bacterial species, i.e. *Enterococcus olivae* (Lucena-Padrós et al., accepted for publication), previously identified

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as *Enterococcus saccharolyticus* (Lucena-Padrós et al., 2014). Although we concluded that Spanish-style green olive fermentation is actually dominated by the species *L. pentosus*, it was also obvious the great biodiversity harbored by this food fermentation. In this study we aimed to further uncover such biodiversity by stepping down to the strain level. For this, and once known their species adscription, we fingerprinted all of the isolates through RAPD and grouped them into phylogenetic dendrograms so that we could examine the microbial population dynamics now at the strain level. It is known that intra-species strain variability is a fact to take into account when examining the actual functionality of a microbial species in a food environment (Ravyts et al., 2012). Such variability dictates the outcome of analyses such as the microbial risk assessment in food (Lianou and Koutsoumanis, 2013) and the evaluation of the biotechnological properties of selected starter cultures (Leroy and De Vuyst, 2004). In particular, in this study we wanted to answer questions such as which strains are ubiquitous in the different fermenters of a particular fermentation yard ("*patio*"), which of them are dominant along the fermentation time, and which of them are shared by different *patios* located far away from each other in the Province of Sevilla. The answers to these questions are of particular importance regarding the dominant species *L. pentosus* as well as those belonging to the LAB group, for it would reveal specific strains of this species with remarkable biotechnological properties useful to improve the output and safety of this food fermentation.

2. Materials and methods

2.1. Origin of the microbial strains and growth conditions

Microbial isolates were obtained from Spanish-style green-olive fermenting brines from two large table-olive manufacturing companies and identified to the species level in a previous work (Lucena-Padrós et al., 2014). These isolates were propagated in their optimal culture media and conditions, as follows. Yeast species were grown aerobically in Glucose–Yeast Extract Agar (OGYE; Mossel et al., 1962) at 30 °C. Bacterial species belonging to the family Enterobacteriaceae were grown in MacConkey Broth Purple (Biokar Diagnostics, Beauvais, France). Bacterial species belonging to the genera *Bacillus*, *Clostridium*, *Paenibacillus*, *Propionibacterium*, *Sporolactobacillus* and *Paracoccus* were grown in Reinforced Clostridial Medium (RCM; Biokar Diagnostics), while those belonging to the genera *Aerococcus*, *Staphylococcus*, *Enterococcus* and *Vibrio* were cultivated in Brain Heart Infusion (BHI; Biokar Diagnostics) supplemented with 0.05% L-cysteine (AppliChem, Darmstadt, Germany). The rest of bacterial species were propagated in de Man–Rogosa–Sharpe (MRS; Biokar Diagnostics) supplemented with 0.05% L-cysteine. Except for *Paracoccus carotinifaciens*, which was grown aerobically at 30 °C, bacteria were cultivated anaerobically at 30 °C using a DG250 Anaerobic Workstation (Don Whitley Scientific Ltd., Shipley, West Yorkshire, UK), with a gas mixture consisting of 10% H_2–10% CO_2–80% N_2. Bacteria used as reference strains (Table 1) for the RAPD-based clustering of strains belonging to the *Lactobacillus plantarum* group were grown in MRS medium, anaerobically at 30 °C.

2.2. Genotyping by randomly amplified polymorphic DNA (RAPD)

Total DNA was extracted directly from colonies by the rapid chloroform method described by Ruiz-Barba et al. (2005). Genotyping was carried out by RAPD using the primer OPL5 (Table 1) as described by Maldonado-Barragán et al. (2013). In the case of cocci, primer ISS1rev (Table 1) was used instead. Amplification products were resolved by electrophoresis through 2% (w/v) agarose gels (SeaKem, Biowhittaker Molecular Applications, USA) in 1× TAE buffer, stained with ethidium bromide (0.5 μg/ml), visualized under UV light and digitally recorded. DNA molecular weight marker 1 kb Plus DNA Ladder (Invitrogen) was used as size standard and as a normalization reference. The resulting RAPD profiles were normalized and analyzed for clustering with the Bionumerics 7.0 software package (Applied Maths, Sint-Martens-Latem, Belgium). Only bands representing amplicons between 150 and 5000 bp in size were included in the analysis. Similarity dendrograms were constructed by the UPGMA clustering method, using the band-based Dice similarity coefficient. The quality of the cluster analysis was verified by calculating the cophenetic correlation value (in percentage) for each dendrogram, using the BioNumerics 7.0 software. Interpretation of values obtained for the similarity coefficients was as follows: 1.0, genetically indistinguishable isolated; 0.99 to 0.80, closely related isolates that are highly similar but not identical, which could be considered the same strain; 0.79 to 0.50, related isolates; <0.50, unrelated isolates (Tenover et al., 1995; Soll, 2000).

Table 1
Bacterial reference strains and oligonucleotides used in this study.

Bacterial strain	Relevant features	Reference
Lactobacillus paraplantarum CNRZ1885^T	From beer contaminant; reference strain[1]	Curk et al. (1996)
Lactobacillus pentosus 128/2[2]	From olive fermentation; reference strain; starter strain[3]	Ruiz-Barba et al. (2010)
L. pentosus LPCO10[2]	From olive fermentation; reference strain; starter strain[3]	Jiménez-Díaz et al. (1993)
Lactobacillus plantarum NC8	From grass silage; reference strain	Aukrust and Blom (1992)
L. plantarum LPT70/3	From olive fermentation; reference strain	Maldonado-Barragán et al. (2013)
Oligonucleotide	**Sequence (5′–3′)**	**Reference**
OPL5	ACGCAGGCAC	Maldonado-Barragán et al. (2013)
ISS1rev	GGATCCAAGACAACGTTTCAAA	Veyrat et al. (1999)

[1] Reference strains were used as internal controls to build the phylogenetic dendrogram of those strains of the *L. plantarum* group.
[2] These strains had been previously described as belonging to the *L. plantarum* species (Jiménez-Díaz et al., 1993), but later identified as *L. pentosus* (Ruiz-Barba et al., 2010; Ruiz-Barba and Jiménez-Díaz, 2012) according to the molecular methods and criteria of Torriani et al. (2001).
[3] Strains that are part of a commercially available starter culture for table olive fermentation.

Reference strains of species belonging to the *L. plantarum* group listed in Table 1 were included in the cluster analysis of the RAPD profiles of bacilli in order to produce an improved distinction among species. As a result, the phylogenetic dendrograms of the bacilli strains were built separately for the *L. plantarum*-group isolates and the non-*L. plantarum*-group ones.

As a control, reproducibility of our PCR fingerprinting experiments was verified with a reduced number of strains. Reproducibility of different RAPD-PCR patterns for the same isolate was always above 80%, which correspond to the minimum level of repeatability for the RAPD fingerprinting technique (Tailliez et al., 1996).

2.3. Biodiversity analyses

Biodiversity of the overall microbial load was evaluated with Margalef's index of genotypes richness (R), Shannon–Weaver's index of diversity (H′) and Simpson's index of dominance (D), calculated as proposed by Ventorino et al. (2007). For strains belonging to the *L. pentosus* species, a strain diversity index (H_1) was calculated for each fermenter by the formula $H_1 = \Sigma P_i \ln P_i$, in which P_i is the relative abundance of each genotype calculated according to the following equation: $P_i = n_i / N$, where n_i was the number of isolates of each genotype and N is the total number of isolates of the same species. Mean values of biodiversity indexes considering time periods were compared through the ANOVA of repeated measures in each *patio*. Comparisons of mean values of biodiversity indexes between *patios* were done by t-Student's test at each fermentation stage. Bartlett and Levene tests were used to check for homogeneity of the variance, while

Kolmogorov–Smirnov test was used to check for normality. A probability value of $P < 0.05$ was regarded to be statistically significant. When it was necessary, values were transformed before the parametric test was carried out. These analyses were performed using the SPSS 21.0 statistical software (SPSS Inc., Chicago, USA). Venn diagrams were drawn using the Venn Diagram Plotter (Pacific Northwest National Laboratory, Richland, WA, U.S.A.).

3. Results

3.1. Bacterial strains diversity

Clustering of the 927 bacterial isolates of this study according to their RAPD profile was carried out separately for bacilli belonging to the *L. plantarum* group, for bacilli not belonging to the *L. plantarum* group, and for cocci.

3.1.1. Strain diversity in the L. plantarum group

The isolates of bacteria belonging to the *L. plantarum* group, a total of 638, could be clustered according to their RAPD profile, using the primer OPL5 and a similarity level of at least 80%, into the 144 different genotypes summarized in Table S1. The isolates belonging to the three species detected, e.g. *L. pentosus*, *L. plantarum* and *Lactobacillus paraplantarum*, could be grouped separately (not shown) in good accordance to the reference strains used (Table 1). Fingerprinting similarity of *L. pentosus* profiles varied from 47% to 100%. With 137 distinct genotypes (Table S1), this species displayed the greatest genotypic variability. This could be the result of the higher number of isolates belonging to this species used for comparison (632). The number of different genotypes identified (91 and 97 from *patios* 1 and 2, respectively) as well as their diversity, measured as the number of isolates per RAPD profile (ca. 3.35), was similar in both *patios*. In spite of the large number of different genotypes of *L. pentosus* involved in olive fermentation at each *patio*, 56% of them (51 genotypes) were actually present in both *patios* (Table S1). Fig. 1 shows the population dynamics, expressed as frequency of isolation, of those *L. pentosus* genotypes present in five or more fermenters along the three fermentation stages in both *patios*. All of these 28 genotypes could be isolated from more than one fermentation stage, and 57% of them were present all along the fermentation (Table S1). Remarkably, we found RAPD profiles of natural isolates similar (≥80%) to the ones exhibited by two of the reference strains used to build the dendrogram, i.e. *L. pentosus* LPCO10 and *L. pentosus* 128/2,

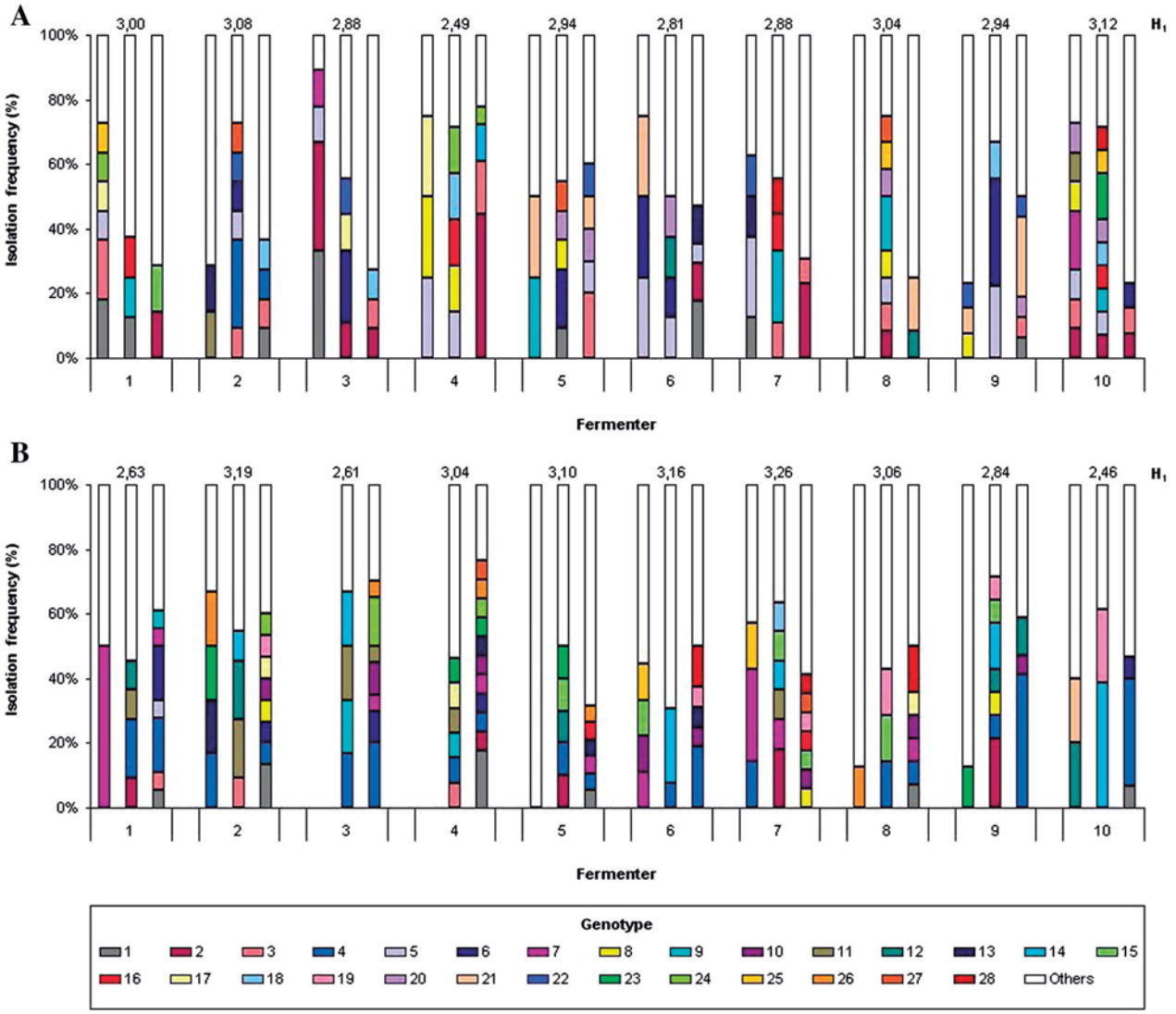

Fig. 1. Population dynamics, expressed as frequency of isolation (%), of the genotypes of *Lactobacillus pentosus* in the ten fermenters of the fermentation yard (*patio*) # 1 (panel A) and #2 (panel B). For each fermenter, from left to right, the three bars represent the genotype frequency at the initial, middle and final stages of fermentation, respectively. Genotype labeled as "Others" represents the grouping of those genotypes which were isolated from less than 5 fermenters, considering both *patios*. H_1: Shannon strain diversity index for each fermenter throughout the fermentation.

corresponding to genotypes nos. 7 and 104 in Table S1, respectively. Actually, isolates highly similar to strain LPCO10 were found in both *patios*, in a total of 9 fermenters (Table S1).

The five isolates identified as *L. plantarum*, along with the two reference strains used, *L. plantarum* NC8 and LPT70/3 (Table 1), were clustered together (not shown) with a similarity level of 59%. Three distinct genotypes could be distinguished among these isolates, all of them from the initial stage of fermentation (Table S1). No strain produced RAPD profiles with a similarity ≥ 80% to any of the reference strains used, i.e. *L. plantarum* NC8 and 70/3. Finally, the only *L. paraplantarum* isolate clustered (not shown) with the reference strain used, i.e. *L. paraplantarum* CNRZ1885^T, at a similarity of 57%.

3.1.2. Strain diversity in the bacilli not-belonging to the L. plantarum group

The isolates of bacilli which did not belong to the *L. plantarum* group, a total of 134, were clustered in a dendrogram using RAPD fingerprinting with primer OPL5 (Fig. S1). Clusters were arbitrarily identified at a similarity level of 50% and resulted in a coherent classification at the species level. These clusters were monospecific, with the exception of that grouping together the species *Clostridium jejuense* and *Clostridium xylanovorans*. On the other hand, the majority of species formed a single cluster, with the exceptions of *Lactobacillus paracollinoides*/*collinoides*, *Paenibacillus illinoisensis*/*xylanilyticus* and *Lactobacillus parafarraginis*. In the two first cases, the two observed clusters could be in accordance with isolates belonging to each of the two possible species forming these groups, although such hypothesis has not been investigated further in our laboratory yet.

Finally, only genotype nos. 5, 10 and 13 of *L. paracollinoides*/*collinoides*, and genotype no. 1 of *Lactobacillus rapi* were isolated from both *patios* (Fig. S1).

3.1.3. Strain diversity in the isolated cocci

Cocci isolates, a total of 155, were clustered in a dendrogram using RAPD fingerprinting with primer ISS1rev (Fig. S2). Preliminary assays of RAPD patterns obtained for this group of isolates showed that primer ISS1rev performed better than OPL5 for identification purposes. The cophenetic correlation value calculated for the dendrogram shown in Fig. S2 was 0.81, indicating a good level of reliability. Cluster analysis of RAPD patterns revealed a high degree of diversity among cocci, which could be separated into nine different groups at an arbitrarily chosen similarity level of 50%. The patterns of genotypes assigned to the same species or that fell into the same major clusters exhibited certain common bands that can be considered representative for the species concerned, except for *E. olivae* (Fig. S2). Isolates of this species grouped in three different clusters, one of them as a subgroup into the cluster containing the *Pediococcus parvulus* isolates, the predominant coccus species in the middle and final stages in *patio* 2, with a similarity coefficient of 62.9%. Isolates identified as *P. parvulus* were genetically distant among them, showing a similarity coefficient of 44.8%, i.e. lower than the similarity level of our arbitrary cluster definition, but they were clearly distinguished from *Pediococcus ethanolidurans* isolates, which was the predominant species of cocci during the middle and final fermentation stages in *patio* 1. Only genotype no. 1 of *P. ethanolidurans* and genotype no. 1 of *Staphylococcus* sp. were isolated from both *patios* (Fig. S2).

3.2. Yeast strains diversity

Clustering in a RAPD-based dendrogram of the 117 yeast strains of this study is shown in Fig. S3. The cophenetic correlation value calculated for this dendrogram was 0.79, indicating a good level of reliability. The twelve yeast species identified in this study grouped well into distinct individual clusters by using the primer OPL5. Nevertheless, discrimination between *Issatchenkia orientalis* and *Kluyveromyces lactis*/*marxianus* isolates was difficult at the similarity degree arbitrarily fixed in this study (50%), most probably due to the similar range of amplified bands obtained for both species (Fig. S3). A similar situation occurred between *Candida parapsilopsis* and *Candida glabrata*, which are actually very related species (Silva et al., 2012). According to their frequency, two species appeared to dominate the Spanish-style olive fermentation: *Saccharomyces cerevisiae* and *Candida thaimueangensis*, which, taken together, represented up to 50% of the total yeast isolates. These species were isolated from virtually all 20 fermenters during the three stages of the fermentation. Four different genotypes of *S. cerevisiae* were isolated, each of them showing to be associated to a precise fermentation stage/s: genotypes nos. 1 and 2 were isolated from the initial and middle stages, while genotypes 3 and 4 were from the middle and final stages, respectively (Fig. S3). Moreover, genotype no. 2 could be isolated from both *patios*, being the only strain isolated from *patio* 2. On the other hand, isolates of the species *C. thaimueangensis* presented less complex (in terms of bands) but more varied genotypes, i.e. up to 7 ones (Fig. S3). Genotype no. 1 represented up to 50% of the isolates and was isolated from 10 different fermenters and, along with genotype no. 6, was isolated from both *patios*. Apart from these, only *Hanseniaspora* sp. showed a genotype which was shared by both *patios*. The rest of species and genotypes were limited to the fermenters of just one of the *patios* examined in this study.

3.3. Biodiversity analyses

The richness, diversity, and dominance indexes of the overall genotypes in both *patios* at the three fermentation stages considered are shown in Fig. 2. No statistical difference in any of these biodiversity indexes was found between both *patios* at any fermentation stage. Fig. 2A shows the evolution of genotype richness, which was stable in *patio* 1 but not in *patio* 2, where a significant difference was observed between the initial and final fermentation stages. Moreover, evolution of diversity (H′) and concentration of dominance (D) indexes were similar in both *patios*, although statistical significance of time effect was detected between the initial and final fermentation stages in both cases (Fig. 2B and C). The highest microbial diversity (1.61 and 1.24 for *patios* 1 and 2, respectively) was found to be associated to the lowest concentration of dominance (0.3 and 0.35 for *patios* 1 and 2, respectively) and characterized the initial fermentation stage (Fig. 2B and C). Conversely, during the middle and final stages, the lowest diversity index values (ca. 0.98) were associated to the highest dominance index values (ca. 0.52) in both *patios* (Fig. 2B and C). Finally, when specifically examining the strain diversity index in the dominant species *L. pentosus* (H_1) in every individual fermenter at both *patios* through fermentation we did not appreciate significant differences in any case (Fig. 1).

3.4. Genotype population dynamics

Dynamics of microbial genotypes isolated along the three fermentation stages of Spanish-style green olive fermentation is represented by proportional Venn diagrams in Fig. 3 for *patios* 1 (panel A) and 2 (panel B). Remarkably, 11 genotypes of the species *L. pentosus* in *patio* 1 and 6 genotypes in *patio* 2 were isolated at the three fermentation stages. In addition, one particular genotype of *Staphylococcus* sp. was also isolated all along the fermentation in *patio* 1. In a previous study (Lucena-Padrós et al., 2014), these isolates could be assigned to the *Staphylococcus epidermidis* group, which is composed by the species *S. epidermidis*, *Staphylococcus saccharoliticus*, *Staphylococcus capitis* and *Staphylococcus caprae* among others, which cannot be easily distinguished just by their 16S rDNA sequence (Kwok and Chow, 2003). Finally, a total of 61 distinct genotypes could be isolated from both *patios*, belonging to 8 different microbial species, 5 bacterial and 3 yeast species (Fig. 4). It is noteworthy the high number of genotypes, a total of 51, of the dominant species *L. pentosus* which were shared by both *patios* (Fig. 4).

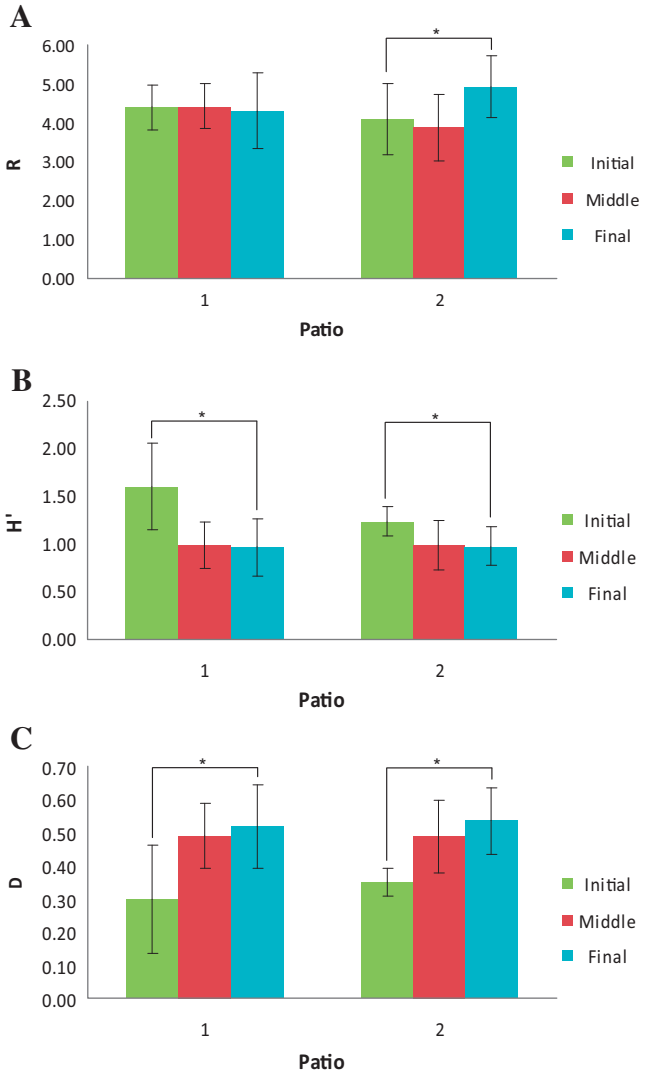

Fig. 2. Richness, diversity and dominance indexes of microbial genotypes at each fermentation stage in two Spanish-style table olive fermentation yards (*patios*). Panel A: Margalef's index of genotype richness (R); Panel B: Shannon–Weaver's index of diversity (H′); Panel C: Simpson's index of dominance (D). Data are shown as mean values with SEM; *Statistically significant difference ($p < 0.05$).

4. Discussion

This work extends, to the strain level, results shown in a previous publication (Lucena-Padrós et al., 2014) on the characteristic species composition of the microbiota related to green olive fermentations at large-scale manufacturing companies. On that occasion, RAPD analysis was used to evaluate the selectivity and discrimination power of culture media used, as well as to control the clonal relationships between isolates from the same samples to avoid repetitive sequencing of the 16S rDNA from the same strains. In this work we have used the same fingerprinting technique to elucidate the intraspecific microbial diversity associated to olive fermentations. This allowed us to investigate also the possible role of specific strains during the fermentations, their distribution among the different fermenters of a given *patio* and assess their "cosmopolitan" character when comparing different *patios*. To do this, RAPD analysis was carried out independently on the different microbial groups previously detected, i.e. yeast, cocci and bacilli belonging and not belonging to the *L. plantarum* group, establishing specific conditions for each group and including, in the case of the dominant *L. plantarum* group, reference strains. Proceeding in this manner, we obtained fairly good grouping of the different strains into the species clusters, allowing further analysis of strain dynamics.

This study has highlighted the microbial diversity at the strain level harbored by the Spanish-style green olive fermentation ecosystem. The fingerprint technique used here allowed fair clustering of the isolates into discrete bacterial or yeast species in the vast majority of the cases. Primer OPL5 was useful in all cases but the cocci isolates, where primer ISS1rev was used instead. In the last case, complexity of the RAPD-PCR band patterns was not uniform for all species identified, and the number of average bands was lower than that obtained for other microbial groups with primer OPL5. In particular, in the case of *P. parvulus* and *E. olivae* the number of bands is too low to make a correct assessment of the actual similarity between isolates. Therefore a polyphasic approach would be recommended in this case. Although it cannot be said for sure that two genotypes sharing ≥80% similarity are the same strain, at least we can say they are closely related by common ancestors (Tailliez et al., 1996).

Most probably influenced by the fact that the isolates in this work were not randomly selected, as we pursued maximal diversity in the morphotypes isolated, high values of genotype richness were achieved. The low number of repeated genotypes per sample indicated that results of species diversity showed in our previous study (Lucena-Padrós et al., 2014) were consistent at the strain (genotype) level too. In this occasion we carried out a global analysis of genetic microbial load in Spanish-style table olive fermentation, including genotypes of both yeast and bacterial species that were previously described as singletons and not taken into consideration for the diversity analyses (Lucena-Padrós et al., 2014). Statistically significant changes in diversity (H′) and concentration of dominance (D) observed in *patio* 1 were mainly due to a loss of genetic diversity associated to the yeast species along the fermentation, which was replaced by an increase of genotype diversity in the predominant bacterial species, i.e. *L. pentosus*. In contrast, in *patio* 2, the observed significant changes in diversity (H′) and dominance (D) values was a consequence of an increase in genotype diversity of the dominant species *L. pentosus* that replaced the loss of diversity associated to *Aerococcus urenaeequi/viridans*, the co-dominant species at the initial fermentation stage in this *patio*. This fact was reinforced by the lost of genotype diversity associated to sporadic species, i.e. those isolated only at this stage.

In a previous work (Lucena-Padrós et al., 2014) it was shown that some microbial species were quite ubiquitous, being present in most of the fermenters examined at each *patio*. Furthermore, a total of 8 bacterial and 3 yeast species were shared by both *patios* studied (Lucena-Padrós et al., 2014). Now we have shown how some precise genotypes persist along the fermentation and even can be isolated from different *patios*, 35 km apart from each other. In particular, Fig. 4 shows that some genotypes belonging to 5 bacterial and 3 yeast species are shared by both *patios*. It is remarkable the number (51) of *L. pentosus* genotypes which could be isolated from both *patios*. Actually, with 137 different (≥80% similarity) RAPD-PCR profiles, the species *L. pentosus* displayed the greatest genotype diversity, although differences in diversity index values (H_1) in the fermenters throughout the fermentation were not significant (Fig. 1). Nevertheless, these facts were no doubt influenced by the large number of isolates obtained of this species (632) in comparison to those obtained from other bacterial or yeasts species. Of special interest are those genotypes which persist all along the fermentation and/or are present in both *patios* (Figs. 3 and 4), for they could display advantageous biotechnological properties of use in olive fermentations. In this sense, up to 16 different *L. pentosus* genotypes were found to persist all along the fermentation (Fig. 3). Also of interest are those genotypes of other 6 LAB species, i.e. *L. paracollinoides/collinoides*, *L. parafarraginis*, *P. ethanolidurans*, *Lactobacillus paracasei*, *L. rapi* and *P. parvulus*, which persist during the middle and final stages (Fig. 3), for they could play an important role in this fermentation which has not been investigated so far.

The fact that the reference strain *L. pentosus* LPCO10 was clustered along with other 13 *L. pentosus* isolates from a total of 9 fermenters at

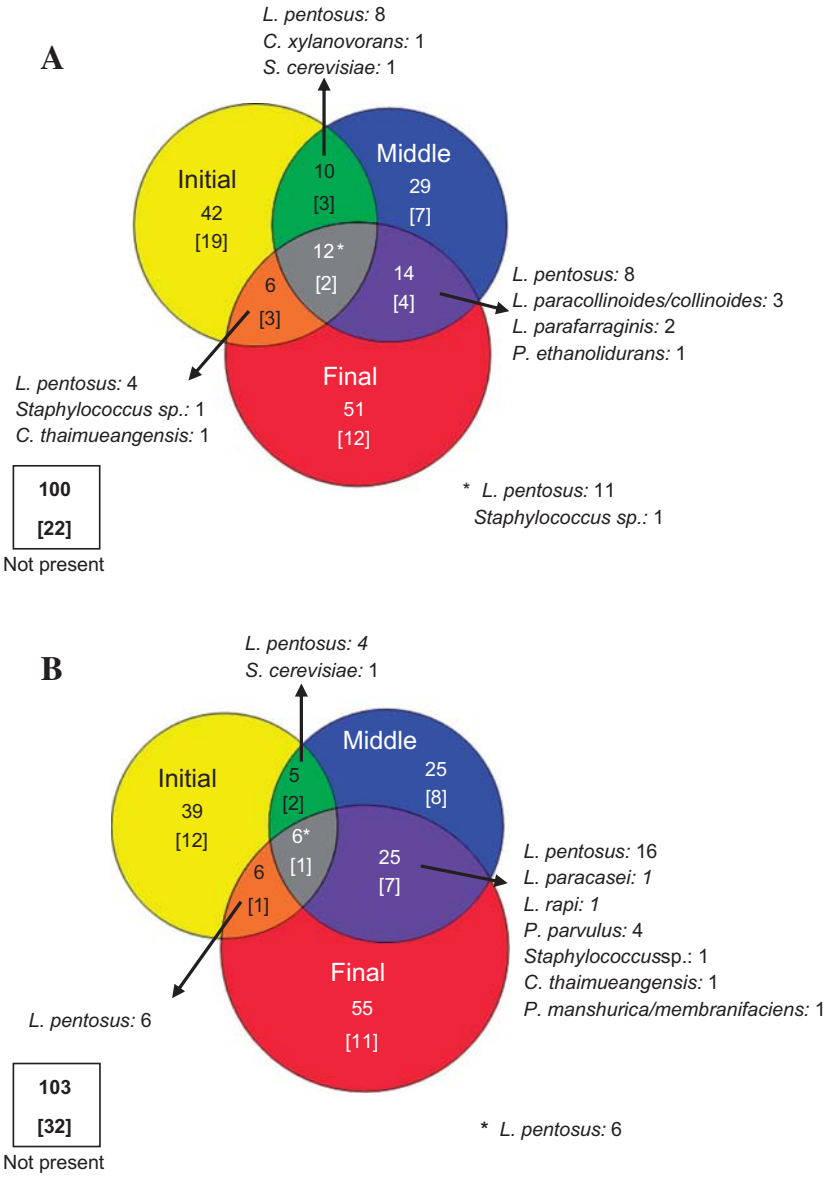

Fig. 3. Microbial genotypes and species distribution along Spanish-style green olive fermentations in *patio* 1 (panel A) and *patio* 2 (panel B). Data are represented by Venn diagrams with proportional areas. The three fermentation stages, initial, middle and final, are indicated along with the number of distinct genotypes which could be isolated at each stage. In brackets are the numbers of different species to which these genotypes belong. For the intersection areas, the actual bacterial and yeast species are indicated. Figures in the square at the left side of each panel indicate the total number of genotypes that were not isolated from that *patio* and the number of microbial species they belong to (in brackets).

both *patios* (Table S1) could be very significative. This strain has been used as part of a commercial starter culture in the *patios* of many companies, although none of the companies studied here used any starter during the season examined here. Nevertheless, personnel at these two companies declared to have used commercial starters based on a mixed culture of *L. pentosus* LPCO10 and *L. pentosus* 128/2 in previous seasons, more specifically during seasons 2007–2008 and 2008–2009 in *patios* 1 and 2, respectively. Also, the strain *L. pentosus* 128/2 could also be clustered along with a single isolate from *patio* 2 (Table S1). As table olive season studied here was 2010–2011 in both *patios*, it suggests that these strains were capable to remain in these *patios* for at least two or three years after deliberate inoculation took place. In the past, we demonstrated that bacteriocin production enhanced dominance and persistence of strains of *L. pentosus* and *L. plantarum* in Spanish-style olive fermentations (Ruiz-Barba et al., 1994, 2010; Ruiz-Barba and Jiménez-Díaz, 2012). Moreover, recent studies showed the ability to form biofilms of the *L. pentosus* strains LPCO10 and 128/2, both on abiotic and biotic surfaces, during Spanish-style olive fermentations (Domínguez-Manzano et al., 2012), including surfaces made of the same material as the fermenter inner walls (Dr. Rufino Jiménez-Díaz, Instituto de la Grasa-CSIC, personal communication). Therefore, although fermenters are emptied and thoroughly cleaned at the end of each season, the inherent porosity of the fermenters (made of glass fiber) along with the ability to form biofilms of some bacterial strains which have grown during the fermentation season, makes it very probable that a characteristic and well adapted microbiota persist season after season in a particular *patio*. Such characteristic microbiota, both at the species as well as the strain levels, could constitute a sort of "fingerprint" of a specific *patio* and therefore could help in the future to early detect problems in the fermentation or to establish the traceability of a table olive batch down to a specific *patio*. This matter is currently being investigated in our laboratory.

It is noteworthy the isolation of one genotype of *Staphylococcus* sp., previously assigned to the *S. epidermidis* group (Lucena-Padrós et al., 2014), which was able to persist all along the olive fermentation and even was shared by both *patios* (Figs. 3 and 4). As some of the species

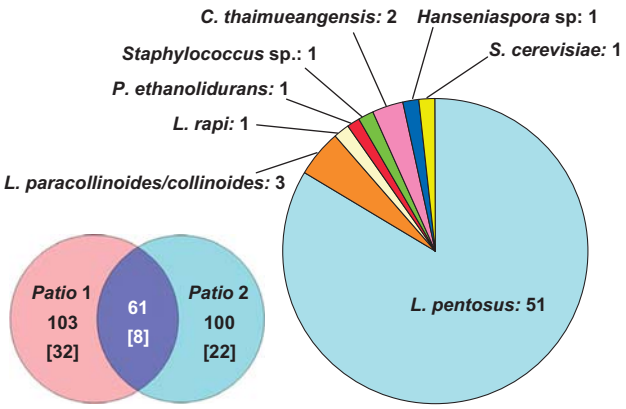

Fig. 4. Number of microbial genotypes, and the species they belong to, shared by both fermentation yards (*patios*) examined in this study during Spanish-style green olive fermentation. The Venn diagram at the left side of the figure indicates the number of genotypes which have only been isolated at each *patio*, along with the number (in brackets) of species they belong to. The intersection of this Venn diagram represents the number of genotypes which are shared by both *patios*, as well as the number (in brackets) of species they belong to.

of this bacterial group have been defined as opportunistic pathogens (Ghebremedhin et al., 2008), this fact poses a note of worry on the existence of unwanted bacteria that appear to be well adapted to this environment. Therefore, it should encourage the necessity of carrying out thorough cleansing of the fermenters before the start of the new season to avoid persistence of some problematic microbial species. On the other hand, such more hygienic practices could prevent from establishing a favorable autochthonous and well adapted microbiota in the fermenters. This could be also the context with newly set up fermenters, especially those located in brand-new *patios*. In both situations, it is therefore advisable the use of appropriate starter cultures based on well adapted strains of LAB and, more specifically, strains of the species *L. pentosus* that contribute to adequate table olive fermentations season after season. In this work we have isolated a wide set of LAB genotypes for this purpose. The strains (genotypes) selected for these starter cultures should persist throughout the fermentation and even after the season finishes. Therefore, they should display good competitive characteristics such as, for instance, bacteriocin production, as it has been demonstrated in the past (Ruiz-Barba et al., 1994; Ruiz-Barba and Jiménez-Díaz, 2012). Finally, it has been stressed the convenience of performing the assessment on the suitability of selected strains to be used for different biotechnological uses under the actual environmental conditions they are intended to be applied (Leroy and De Vuyst, 2004). Therefore, isolation and preservation of wild-type strains from traditional products should be encouraged. This study was aimed to contribute to this goal.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2014.07.035.

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References

AAO, 2013. Agencia para el Aceite de Oliva [Olive Oil Agency], Market Information. Available at: http://aplicaciones.magrama.es/pwAgenciaAO/InfGeneral.aao?opcion_seleccionada=1000&control_acceso=S&idioma=ING (accessed the 6th of February, 2013).

Aukrust, T., Blom, H., 1992. Transformation of *Lactobacillus* strains used in meat and vegetable fermentations. Food Res. Int. 25, 253–261.

Botta, C., Cocolin, L., 2012. Microbial dynamics and biodiversity in table olive fermentation: culture-dependent and -independent approaches. Front. Microbiol. 3, 245.

Curk, M.-C., Hubert, J.-C., Bringel, F., 1996. *Lactobacillus paraplantarum* sp. nov., a new species related to *Lactobacillus plantarum*. Int. J. Syst. Bacteriol. 46, 595–598.

De Castro, A., Montaño, A., Casado, F.-J., Sánchez, A.-H., Rejano, L., 2002. Utilization of *Enterococcus casseliflavus* and *Lactobacillus pentosus* as starter culture for Spanish-style green olive fermentation. Food Microbiol. 19, 637–644.

Domínguez-Manzano, J., Olmo-Ruiz, C., Bautista-Gallego, J., Arroyo-López, F.N., Garrido-Fernández, A., Jiménez-Díaz, R., 2012. Biofilm formation on abiotic and biotic surfaces during Spanish style green table olive fermentation. Int. J. Food Microbiol. 157, 230–238.

Garrido Fernández, A., Fernández Díez, M.J., Adams, R.M., 1997. Table Olives: Production and Processing. Chapman & Hall, London, pp. 134–197.

Ghebremedhin, B., Layer, F., König, W., König, B., 2008. Genetic classification and distinguishing of *Staphylococcus* species based on different partial gap, 16S rRNA, hsp60, rpoB, sodA, and tuf gene sequences. J. Clin. Microbiol. 46, 1019–1025.

Jiménez-Díaz, R., Rios-Sánchez, R.M., Desmazeaud, M., Ruiz-Barba, J.L., Piard, J.C., 1993. Plantaricin S and T, two new bacteriocins produced by *Lactobacillus plantarum* LPCO10 isolated from a green olive fermentation. Appl. Environ. Microbiol. 59, 1416–1424.

Kwok, A.Y., Chow, A.W., 2003. Phylogenetic study of *Staphylococcus* and *Macrococcus* species based on partial hsp60 gene sequences. Int. J. Syst. Evol. Microbiol. 53, 87–92.

Leroy, F., De Vuyst, L., 2004. Lactic acid bacteria as functional starter cultures for the food fermentation industry. Trends Food Sci. Technol. 15, 67–78.

Lianou, A., Koutsoumanis, K.P., 2013. Strain variability of the behavior of foodborne bacterial pathogens: a review. Int. J. Food Microbiol. 167, 310–321.

Lucena-Padrós, H., Caballero-Guerrero, B., Maldonado-Barragán, A., Ruiz-Barba, J.L., 2014. Microbial diversity and dynamics of Spanish-style green table-olive fermentations in large manufacturing companies through culture-dependent techniques. Food Microbiol. 42, 154–165.

Lucena-Padrós, H., González, J.M., Caballero-Guerrero, B., Ruiz-Barba, J.L., Maldonado-Barragán, A., 2014. *Enterococcus olivae* sp. nov., isolated from Spanish-style green-olive fermentations. Int. J. Syst. Evol. Microbiol. 64, 2534–2539.

Maldonado-Barragán, A., Caballero-Guerrero, B., Lucena-Padrós, H., Ruiz-Barba, J.L., 2013. Induction of bacteriocin production by coculture is widespread among plantaricin-producing *Lactobacillus plantarum* strains with different regulatory operons. Food Microbiol. 33, 40–47.

Mossel, D.A.A., Visser, M., Mengerink, W.H.J., 1962. A comparison of media for the enumeration of moulds and yeasts in foods and beverages. Lab. Pract. 11, 109–112.

Ravyts, F., Vuyst, L.D., Leroy, F., 2012. Bacterial diversity and functionalities in food fermentations. Eng. Life Sci. 12, 356–367.

Rejano, L., Montaño, A., Casado, F.J., Sánchez, A.H., de Castro, A., 2010. Table olives: varieties and variations. In: Preedy, V.R., Watson, R.R. (Eds.), Olives and Olive Oil in Health and Disease Prevention. Academic Press, Oxford, pp. 5–15.

Ruiz-Barba, J.L., Jiménez-Díaz, R., 2012. A novel *Lactobacillus pentosus*-paired starter culture for Spanish-style green olive fermentation. Food Microbiol. 30, 253–259.

Ruiz-Barba, J.L., Cathcart, D.P., Warner, P.J., Jiménez-Díaz, R., 1994. Use of *Lactobacillus plantarum* LPCO10, a bacteriocin producer, as a starter culture in Spanish-style green olive fermentations. Appl. Environ. Microbiol. 60, 2059–2064.

Ruiz-Barba, J.L., Maldonado, A., Jiménez-Díaz, R., 2005. Small-scale total DNA extraction from bacteria and yeast for PCR applications. Anal. Biochem. 347, 333–335.

Ruiz-Barba, J.L., Caballero-Guerrero, B., Maldonado-Barragán, A., Jiménez-Díaz, R., 2010. Coculture with specific inducing bacteria notably enhances survival of the autoinducer-regulated bacteriocin producer *Lactobacillus plantarum* NC8 in olive fermentations. Food Microbiol. 27, 413–417.

Silva, S., Negri, M., Henriques, M., Oliveira, R., Williams, D.W., Azeredo, J., 2012. *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*: biology, epidemiology, pathogenicity and antifungal resistance. FEMS Microbiol. Rev. 36, 288–305.

Soll, D.R., 2000. The ins and outs of DNA fingerprinting the infectious fungi. Clin. Microbiol. Rev. 13, 332–370.

Tailliez, P., Quénée, P., Chopin, A., 1996. Estimation de la diversité parmi les souches de la collection CNRZ: application de la RAPD à un groupe de lactobacilles. Lait 76, 147–158.

Tenover, F.C., Arbeit, R.D., Goering, R.V., Mickelsen, P.A., Murray, B.E., Persing, D.H., Swaminathan, B., 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial typing. J. Clin. Microbiol. 33, 2233–2239.

Torriani, S., Felis, G.E., Dellaglio, F., 2001. Differentiation of *Lactobacillus plantarum*, *L. pentosus*, and *L. paraplantarum* by *recA* gene sequence analysis and multiplex PCR assay with *recA* gene-derived primers. Appl. Environ. Microbiol. 67, 3450–3454.

Ventorino, V., Chiurazzi, M., Aponte, M., Pepe, O., Moschetti, G., 2007. Genetic diversity of a natural population of *Rhizobium leguminosarum* bv. *viciae* nodulating plants of *Vicia faba* in the Vesuvian area. Curr. Microbiol. 55, 512–517.

Veyrat, A., Miralles, M.C., Pérez-Martínez, G., 1999. A fast method for monitoring the colonization rate of lactobacilli in a meat model system. J. Appl. Microbiol. 87, 49–61.

Table S1. Strain diversity in the species of the *Lactobacillus plantarum* group isolated from Spanish-style green olive fermentations.

Species	Genotype[1]	Isolates[2]	*Patio*[3]	Fermenters[4]	Stage[5]
Lactobacillus paraplantarum	1	1	2	1	I
Lactobacillus plantarum	1	2	1	1	I
Lactobacillus plantarum	2	1	1	1	I
Lactobacillus plantarum	3	2	1	1	I
Lactobacillus pentosus	1	22	1;2	13	I;M;F
Lactobacillus pentosus	2	32	1;2	12	I;M;F
Lactobacillus pentosus	3	19	1;2	12	I;M;F
Lactobacillus pentosus	4	40	1;2	11	I;M;F
Lactobacillus pentosus	5	17	1;2	11	I;M;F
Lactobacillus pentosus	6	19	1;2	11	M;F
Lactobacillus pentosus	7[6]	13	1;2	9	I;M;F
Lactobacillus pentosus	8	9	1;2	8	I;M;F
Lactobacillus pentosus	9	10	1;2	8	I;M;F
Lactobacillus pentosus	10	9	2	7	I;F
Lactobacillus pentosus	11	9	1;2	7	I;M;F
Lactobacillus pentosus	12	10	1;2	7	I;M;F
Lactobacillus pentosus	13	8	1;2	7	I;F
Lactobacillus pentosus	14	15	1;2	7	M;F
Lactobacillus pentosus	15	8	1;2	6	I;M;F
Lactobacillus pentosus	16	6	1;2	6	M;F
Lactobacillus pentosus	17	6	1;2	6	I;M;F
Lactobacillus pentosus	18	6	1;2	6	M;F
Lactobacillus pentosus	19	8	2	6	M;F
Lactobacillus pentosus	20	7	1	5	I;M;F
Lactobacillus pentosus	21	11	1;2	5	I;F
Lactobacillus pentosus	22	6	1	5	I;M;F
Lactobacillus pentosus	23	7	1;2	5	I;M;F
Lactobacillus pentosus	24	8	1;2	5	I;M;F
Lactobacillus pentosus	25	5	1;2	5	I;M
Lactobacillus pentosus	26	5	2	5	I;F
Lactobacillus pentosus	27	5	1;2	5	M;F
Lactobacillus pentosus	28	7	1;2	5	M;F
Lactobacillus pentosus	29	9	2	4	I;M;F
Lactobacillus pentosus	30	7	1;2	4	I;M;F
Lactobacillus pentosus	31	7	1;2	4	I;M;F
Lactobacillus pentosus	32	4	2	4	I
Lactobacillus pentosus	33	4	1;2	4	M;F
Lactobacillus pentosus	34	4	2	4	M;F
Lactobacillus pentosus	35	4	1;2	4	M;F
Lactobacillus pentosus	36	4	1;2	4	I;M;F
Lactobacillus pentosus	37	4	1;2	4	M;F
Lactobacillus pentosus	38	4	1;2	4	I;F
Lactobacillus pentosus	39	4	1;2	4	M;F
Lactobacillus pentosus	40	5	2	4	F
Lactobacillus pentosus	41	5	2	4	M;F
Lactobacillus pentosus	42	5	1;2	4	M;F
Lactobacillus pentosus	43	7	1;2	4	F
Lactobacillus pentosus	44	4	1;2	4	I;F
Lactobacillus pentosus	45	4	1	3	I;M;F

Lactobacillus pentosus	46	5	1	3	I;M
Lactobacillus pentosus	47	3	1;2	3	F
Lactobacillus pentosus	48	3	1;2	3	I;F
Lactobacillus pentosus	49	3	1	3	I;M
Lactobacillus pentosus	50	3	1;2	3	M;F
Lactobacillus pentosus	51	3	1;2	3	F
Lactobacillus pentosus	52	3	2	3	M;F
Lactobacillus pentosus	53	3	1	3	I;M;F
Lactobacillus pentosus	54	4	2	3	I;F
Lactobacillus pentosus	55	4	1	3	F
Lactobacillus pentosus	56	4	1;2	3	I;M;F
Lactobacillus pentosus	57	4	1;2	3	I;F
Lactobacillus pentosus	58	4	1;2	3	F
Lactobacillus pentosus	59	4	1	3	M
Lactobacillus pentosus	60	5	1;2	3	M;F
Lactobacillus pentosus	61	5	1	3	F
Lactobacillus pentosus	62	6	1	3	F
Lactobacillus pentosus	63	3	1	2	I;M
Lactobacillus pentosus	64	3	1	2	M;F
Lactobacillus pentosus	65	3	1	2	M;F
Lactobacillus pentosus	66	2	2	2	M
Lactobacillus pentosus	67	2	2	2	M;F
Lactobacillus pentosus	68	2	1	2	M
Lactobacillus pentosus	69	2	1	2	I
Lactobacillus pentosus	70	2	2	2	F
Lactobacillus pentosus	71	2	2	2	I;M
Lactobacillus pentosus	72	2	2	2	I;M
Lactobacillus pentosus	73	2	1	2	M;F
Lactobacillus pentosus	74	2	2	2	F
Lactobacillus pentosus	75	2	1	2	I;F
Lactobacillus pentosus	76	2	1;2	2	F
Lactobacillus pentosus	77	2	2	2	I;M
Lactobacillus pentosus	78	2	1;2	2	I;M
Lactobacillus pentosus	79	2	1;2	2	M;F
Lactobacillus pentosus	80	2	2	2	I
Lactobacillus pentosus	81	2	1;2	2	I;F
Lactobacillus pentosus	82	2	1;2	2	M;F
Lactobacillus pentosus	83	2	1;2	2	M;F
Lactobacillus pentosus	84	2	2	2	F
Lactobacillus pentosus	85	2	1;2	2	F
Lactobacillus pentosus	86	2	1;2	2	M;F
Lactobacillus pentosus	87	2	1	2	I;M
Lactobacillus pentosus	88	2	1	2	M
Lactobacillus pentosus	89	2	1	2	F
Lactobacillus pentosus	90	3	2	2	M;F
Lactobacillus pentosus	91	3	1	2	I
Lactobacillus pentosus	92	3	1	2	F
Lactobacillus pentosus	93	3	1	2	F
Lactobacillus pentosus	94	4	2	2	M
Lactobacillus pentosus	95	4	1;2	2	M;F
Lactobacillus pentosus	96	6	2	2	M
Lactobacillus pentosus	97	6	1	2	I
Lactobacillus pentosus	98	7	2	2	F
Lactobacillus pentosus	99	1	1	1	M

Lactobacillus pentosus	100	1	2	1	F
Lactobacillus pentosus	101	1	2	1	F
Lactobacillus pentosus	102	1	2	1	M
Lactobacillus pentosus	103	1	2	1	F
Lactobacillus pentosus	104[7]	1	2	1	M
Lactobacillus pentosus	105	1	1	1	M
Lactobacillus pentosus	106	1	2	1	I
Lactobacillus pentosus	107	1	1	1	M
Lactobacillus pentosus	108	1	2	1	M
Lactobacillus pentosus	109	1	1	1	F
Lactobacillus pentosus	110	1	2	1	M
Lactobacillus pentosus	111	1	1	1	M
Lactobacillus pentosus	112	1	2	1	I
Lactobacillus pentosus	113	1	2	1	I
Lactobacillus pentosus	114	1	1	1	F
Lactobacillus pentosus	115	1	1	1	I
Lactobacillus pentosus	116	1	1	1	F
Lactobacillus pentosus	117	1	2	1	I
Lactobacillus pentosus	118	1	2	1	F
Lactobacillus pentosus	119	1	2	1	F
Lactobacillus pentosus	120	1	2	1	I
Lactobacillus pentosus	121	1	1	1	F
Lactobacillus pentosus	122	1	1	1	F
Lactobacillus pentosus	123	1	2	1	F
Lactobacillus pentosus	124	1	2	1	I
Lactobacillus pentosus	125	1	1	1	I
Lactobacillus pentosus	126	1	1	1	F
Lactobacillus pentosus	127	1	1	1	F
Lactobacillus pentosus	128	2	2	1	F
Lactobacillus pentosus	129	2	2	1	F
Lactobacillus pentosus	130	2	2	1	I
Lactobacillus pentosus	131	2	1	1	F
Lactobacillus pentosus	132	2	1	1	F
Lactobacillus pentosus	133	3	2	1	F
Lactobacillus pentosus	134	3	2	1	M
Lactobacillus pentosus	135	4	2	1	F
Lactobacillus pentosus	136	4	2	1	F
Lactobacillus pentosus	137	5	1	1	F

[1]Genotyping is based on RAPD profiles, and it is the result of clustering isolates exhibiting more than 80% similarity.
[2]Number of isolates belonging to a distinct genotype.
[3]Indicates from which fermentation yard (*patio*) that genotype could be isolated.
[4]Number of fermenters, out of a total of 20, from which that genotype could be isolated.
[5]Fermentation stages at which that genotype could be isolated. I, initial; M, Middle; F, Final.
[6]The reference strain *L. pentosus* LPCO10 clustered in this RAPD profile with ≥80% similarity.
[7] The reference strain *L. pentosus* 128/2 clustered in this RAPD profile with ≥80% similarity.

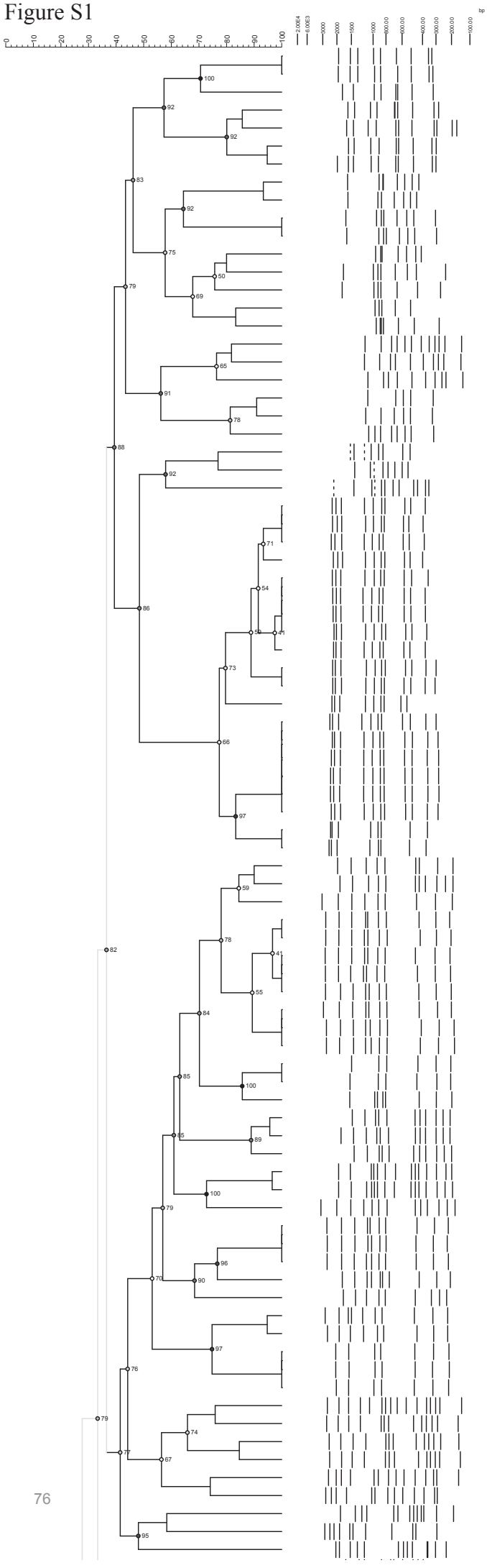
Supplementary material - Figure Legends

Figure S1. Phylogenetic dendrogram obtained from RAPD-PCR profiles with primer OPL5 of 134 isolates of bacilli which did not belong to the *L. plantarum* group during Spanish-style green olive fermentations from two different fermentation yards (*patios*). The number of fermenters, out of a total of 20, from which a particular genotype could be isolated is indicated in the column labelled "Fermenter". The fermentation stage at which a particular genotype could be isolated is indicated in the column labelled "Stage": I, initial; M, Middle; F, Final. Scale line at the top indicates the percentage of similarity. The 1 Kb Plus DNA ladder (Invitrogen) used to normalize banding patterns is represented at the top of the figure.

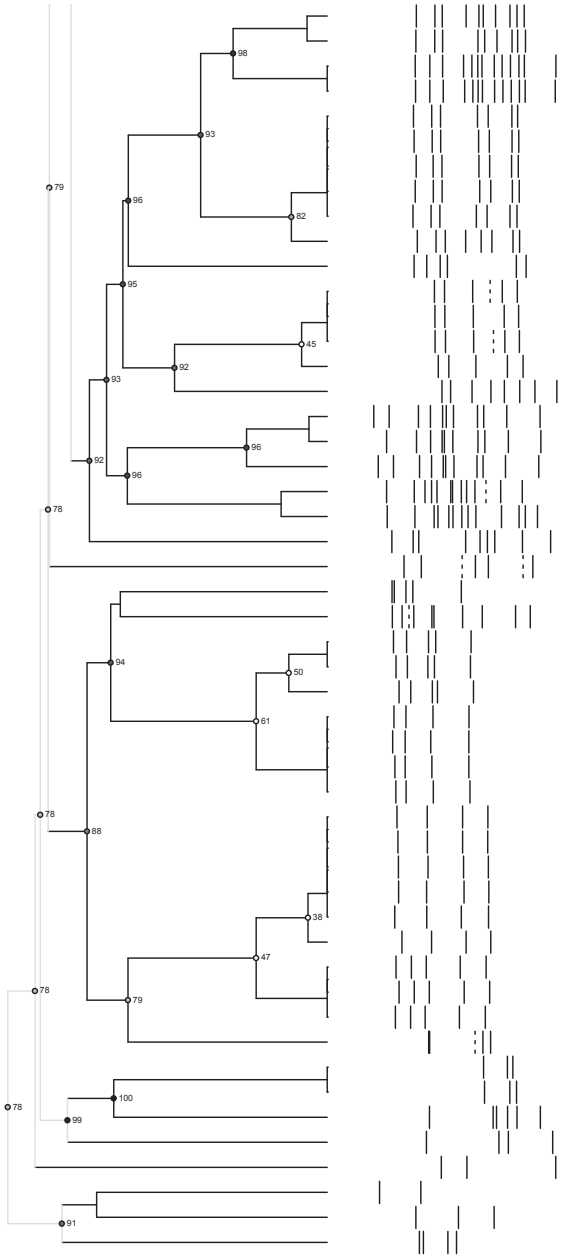
Figure S2. Phylogenetic dendrogram obtained from RAPD-PCR profiles with primer ISS1rev of 155 isolates of cocci during Spanish-style green olive fermentations from two different fermentation yards (*patios*). The number of fermenters, out of a total of 20, from which a particular genotype could be isolated is indicated in the column labelled "Fermenter". The fermentation stage at which a particular genotype could be isolated is indicated in the column labelled "Stage": I, initial; M, Middle; F, Final. Scale line at the top indicates the percentage of similarity. The 1 Kb Plus DNA ladder (Invitrogen) used to normalize banding patterns is represented at the top of the figure.

Figure S3. Phylogenetic dendrogram obtained from RAPD-PCR profiles with primer OPL5 of 117 yeast isolates during Spanish-style green olive fermentations from two different fermentation yards (*patios*). The number of fermenters, out of a total of 20, from which a particular genotype could be isolated is indicated in the column labelled "Fermenter". The fermentation stage at which a particular genotype could be isolated is indicated in the column labelled "Stage": I, initial; M, Middle; F, Final. Scale line at the top indicates the percentage of similarity. The 1 Kb Plus DNA ladder (Invitrogen) used to normalize banding patterns is represented at the top of the figure.

Figure S1



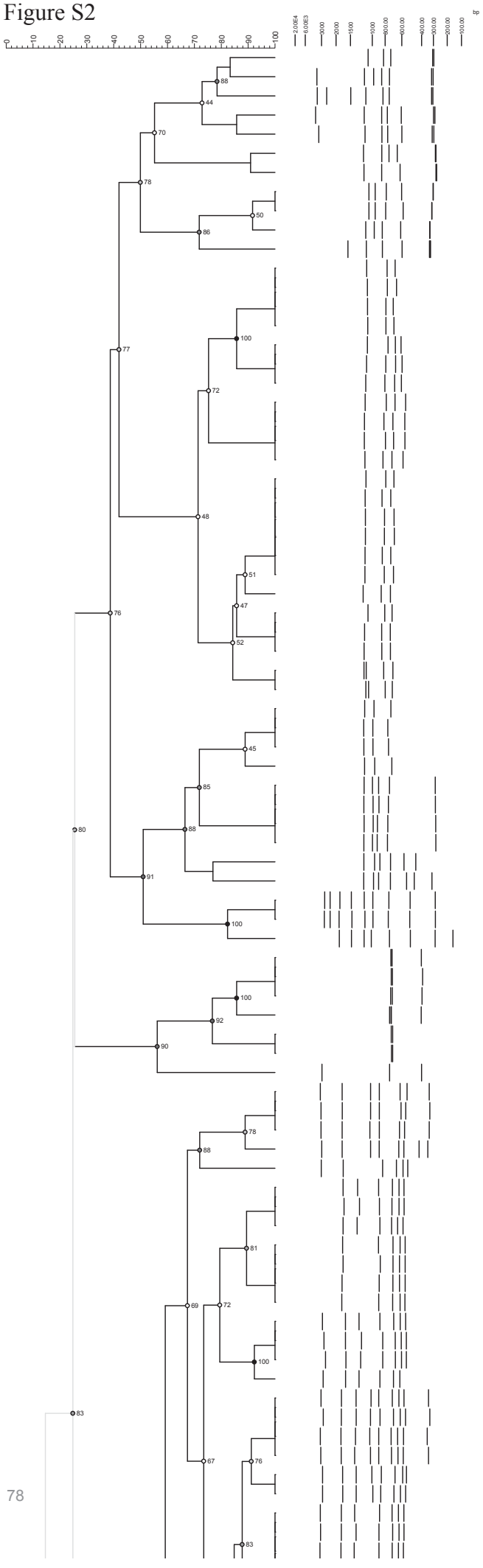
Identification	Isolate	Genotype	Patio	Fermenter	Stage
Lactobacillus paracollinoides/collinoides	J50.8B	1	1	6	F
Lactobacillus paracollinoides/collinoides	J50.9B	1	1	6	F
Lactobacillus paracollinoides/collinoides	J41.3B	2	1	2	F
Lactobacillus paracollinoides/collinoides	J28.5	3	1	3	M
Lactobacillus paracollinoides/collinoides	J45.3	3	1	5	F
Lactobacillus paracollinoides/collinoides	J42.5	3	1	8	F
Lactobacillus paracollinoides/collinoides	J45.7B	3	1	5	F
Lactobacillus parafarraginis	J34.3	1	1	7	M
Lactobacillus parafarraginis	J31.6	1	1	4	M
Lactobacillus parafarraginis	J48.6B	2	1	3	F
Lactobacillus parafarraginis	J50.1	2	1	6	F
Lactobacillus parafarraginis	J35.4	3	1	1	M
Lactobacillus parafarraginis	J50.6B	3	1	6	F
Lactobacillus parafarraginis	J45.5	4	1	5	F
Lactobacillus parafarraginis	J33.3	5	1	10	M
Lactobacillus parafarraginis	J45.8B	5	1	5	F
Lactobacillus rapi	J54.2B	1	1	7	F
Lactobacillus rapi	G32.4	1	2	9	M
Lactobacillus rapi	G40.4	2	2	5	M
Lactobacillus rapi	G40.2	3	2	5	M
Lactobacillus rapi	G51.2B	3	2	10	F
Lactobacillus rapi	G58.3B	3	2	3	F
Paenibacillus sp.	G11.31	1	2	10	I
Paenibacillus sp.	G17.33	2	2	2	I
Paenibacillus illinoisensis/xylanilyticus	G16.31	1	2	1	I
Lactobacillus paracasei	G33.12	1	2	6	M
Lactobacillus paracasei	G34.12	1	2	7	M
Lactobacillus paracasei	G35.12	1	2	8	M
Lactobacillus paracasei	G33.13	1	2	6	M
Lactobacillus paracasei	G34.2	1	2	7	M
Lactobacillus paracasei	G36.14	1	2	1	M
Lactobacillus paracasei	G37.12	1	2	2	M
Lactobacillus paracasei	G60.3B	1	2	5	F
Lactobacillus paracasei	G35.13	1	2	8	M
Lactobacillus paracasei	G35.1B	1	2	8	M
Lactobacillus paracasei	G40.8	1	2	5	M
Lactobacillus paracasei	G60.4	2	2	5	F
Lactobacillus paracasei	G33.2B	3	2	6	M
Lactobacillus paracasei	G34.10	3	2	7	M
Lactobacillus paracasei	G35.2	3	2	8	M
Lactobacillus paracasei	G35.7	3	2	8	M
Lactobacillus paracasei	G35.8	3	2	8	M
Lactobacillus paracasei	G35.10	3	2	8	M
Lactobacillus paracasei	G33.5	3	2	6	M
Lactobacillus paracasei	G31.12	3	2	10	M
Lactobacillus paracollinoides/collinoides	J30.1	4	1	6	M
Lactobacillus paracollinoides/collinoides	J46.9B	4	1	9	F
Lactobacillus paracollinoides/collinoides	J30.14	4	1	6	M
Lactobacillus paracollinoides/collinoides	J28.4	5	1	3	M
Lactobacillus paracollinoides/collinoides	J35.3	5	1	1	M
Lactobacillus paracollinoides/collinoides	J26.6	5	1	9	M
Lactobacillus paracollinoides/collinoides	J30.2	5	1	6	M
Lactobacillus paracollinoides/collinoides	J31.2	5	1	4	M
Lactobacillus paracollinoides/collinoides	J46.8B	5	1	9	F
Lactobacillus paracollinoides/collinoides	G59.3B	5	2	4	F
Lactobacillus paracollinoides/collinoides	G52.1	5	2	9	F
Lactobacillus paracollinoides/collinoides	J21.2	6	1	2	M
Lactobacillus paracollinoides/collinoides	J25.3	6	1	5	M
Lactobacillus paracollinoides/collinoides	J28.6	6	1	3	M
Lactobacillus paracollinoides/collinoides	J41.4	7	1	2	F
Lactobacillus paracollinoides/collinoides	J41.6	7	1	2	F
Lactobacillus paracollinoides/collinoides	J50.4	7	1	6	F
Lactobacillus paracollinoides/collinoides	J21.5	8	1	2	M
Lactobacillus paracollinoides/collinoides	J30.3	8	1	6	M
Lactobacillus paracollinoides/collinoides	J46.2	9	1	9	F
Lactobacillus paracollinoides/collinoides	J31.4	10	1	4	M
Lactobacillus paracollinoides/collinoides	J31.9	10	1	4	M
Lactobacillus paracollinoides/collinoides	G55.4	10	2	8	F
Lactobacillus paracollinoides/collinoides	J25.4	11	1	5	M
Lactobacillus paracollinoides/collinoides	J41.4B	12	1	2	F
Lactobacillus paracollinoides/collinoides	J22.4	13	1	8	M
Lactobacillus paracollinoides/collinoides	G51.1	13	2	10	F
Lactobacillus paracollinoides/collinoides	J21.9	14	1	2	M
Lactobacillus paracollinoides/collinoides	J22.1	14	1	8	M
Lactobacillus paracollinoides/collinoides	J22.2	14	1	8	M
Lactobacillus rhamnosus	G32.8	1	2	9	M
Lactobacillus rhamnosus	G57.6	2	2	2	F
Lactobacillus rhamnosus	G57.2	3	2	2	F
Lactobacillus rhamnosus	G59.4	3	2	4	F
Lactobacillus rhamnosus	G37.1B	4	2	2	M
Lactobacillus rhamnosus	G57.4B	5	2	2	F
Enterobacter hormaechei	J11.12	1	1	4	I
Escherichia coli	G20.17	1	2	5	I
Paracoccus carotinifaciens	G19.31	1	2	4	I



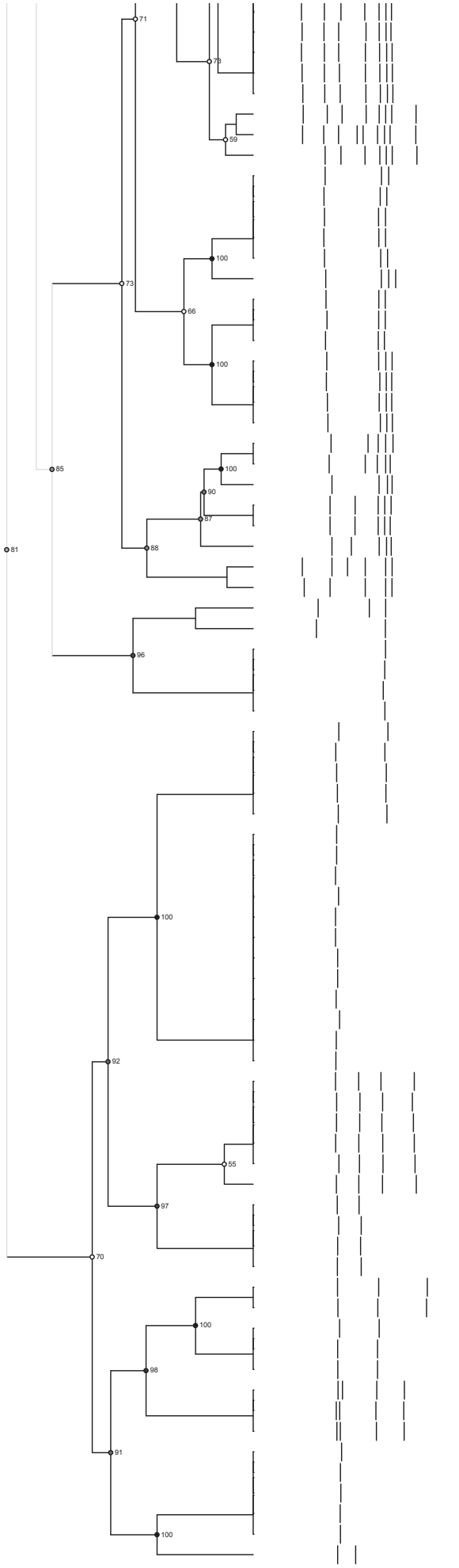
Lactobacillus coryniformis	G38.8	1	2	3	M
Lactobacillus coryniformis	G37.13	1	2	2	M
Lactobacillus coryniformis	G38.12	2	2	3	M
Lactobacillus coryniformis	G40.14	2	2	5	M
Lactobacillus coryniformis	G53.2B	3	2	6	F
Lactobacillus coryniformis	G54.2	3	2	7	F
Lactobacillus coryniformis	G54.4	3	2	7	F
Lactobacillus coryniformis	G54.5	3	2	7	F
Lactobacillus coryniformis	G54.1B	3	2	7	F
Lactobacillus coryniformis	G53.8	3	2	6	F
Paenibacillus illinoisensis/xylanilyticus	J30.31	2	1	6	M
Clostridium xylanovorans	J6.31	1	1	9	I
Clostridium xylanovorans	J10.31	1	1	6	I
Clostridium xylanovorans	J13.31	1	1	10	I
Clostridium xylanovorans	J35.31	1	1	1	M
Clostridium jejuense	J14.32	1	1	7	I
Propionibacterium acnes	J41.32	1	1	2	F
Propionibacterium acnes	J41.33	1	1	2	F
Propionibacterium acnes	J41.31	2	1	2	F
Sporolactobacillus inulinus/terrae	G31.31	1	2	10	M
Sporolactobacillus inulinus/terrae	G31.32	1	2	10	M
Enterobacter kobei	G18.15	1	2	3	I
Enterobacter radicincitans/oryzae	J1.4	1	1	2	I
Lactobacillus parafarraginis	J50.7B	6	1	6	F
Yersinia enterocolitica subsp. enterocolitica	G16.42	1	2	1	I
Weissella paramesenteroides/hellenica	J11.1	1	1	4	I
Weissella paramesenteroides/hellenica	J15.7	1	1	1	I
Weissella paramesenteroides/hellenica	J15.10	1	1	1	I
Weissella paramesenteroides/hellenica	J1.2	1	1	2	I
Weissella paramesenteroides/hellenica	J2.2	1	1	8	I
Weissella paramesenteroides/hellenica	J5.3	1	1	5	I
Weissella paramesenteroides/hellenica	J10.1	1	1	6	I
Vibrio furnissii/fluvialis	J2.11v	1	1	8	I
Vibrio furnissii/fluvialis	J2.12v	1	1	8	I
Vibrio furnissii/fluvialis	J5.11v	1	1	5	I
Vibrio furnissii/fluvialis	J5.12v	1	1	5	I
Vibrio furnissii/fluvialis	J8.14v	1	1	3	I
Vibrio furnissii/fluvialis	J15.13v	1	1	1	I
Vibrio furnissii/fluvialis	J1.11v	1	1	2	I
Vibrio furnissii/fluvialis	J8.11v	1	1	3	I
Vibrio furnissii/fluvialis	J10.11v	1	1	6	I
Clostridium sartagoforme	J15.31	1	1	1	I
Escherichia sp.	J11.2	1	1	4	I
Escherichia sp.	J11.11	1	1	4	I
Bacillus circulans	J5.31	1	1	5	I
Clostridium schirmacherense/argentinense	J8.31	1	1	3	I
Enterobacter sp.	J11.44	1	1	4	I
Bacillus weihenstephanensis/mycoides	J33.31	1	1	10	M
Lactobacillus parafarraginis	G51.1B	7	2	10	F
Pantoea agglomerans	J55.13	1	1	1	F

Figure S1

Figure S2



Identification	**Isolate**	**Genotype**	**Patio**	**Fermenter**	**Stage**
Enterococcus casseliflavus	J2.12	1	1	8	I
Enterococcus casseliflavus	J8.43	1	1	3	I
Enterococcus casseliflavus	J5.6	2	1	5	I
Enterococcus casseliflavus	J10.5	3	1	6	I
Enterococcus casseliflavus	J2.43	3	1	8	I
Enterococcus casseliflavus	J10.3	4	1	6	I
Enterococcus casseliflavus	J10.43	4	1	6	I
Enterococcus casseliflavus	J8.10	5	1	3	I
Enterococcus casseliflavus	J2.44	5	1	8	I
Enterococcus casseliflavus	J2.13	5	1	8	I
Enterococcus casseliflavus	J1.9	6	1	2	I
Pediococcus ethanolidurans	J42.10	1	1	8	F
Pediococcus ethanolidurans	J48.1	1	1	3	F
Pediococcus ethanolidurans	J42.2B	1	1	8	F
Pediococcus ethanolidurans	J45.2	1	1	5	F
Pediococcus ethanolidurans	J42.2	1	1	8	F
Pediococcus ethanolidurans	G52.1B	1	2	9	F
Pediococcus ethanolidurans	G52.5B	1	2	9	F
Pediococcus ethanolidurans	J45.10	2	1	5	F
Pediococcus ethanolidurans	J45.2B	2	1	5	F
Pediococcus ethanolidurans	J45.5B	2	1	5	F
Pediococcus ethanolidurans	J45.6B	2	1	5	F
Pediococcus ethanolidurans	J25.5	3	1	5	M
Pediococcus ethanolidurans	J25.10	3	1	5	M
Pediococcus ethanolidurans	J42.4	3	1	8	F
Pediococcus ethanolidurans	J46.7B	3	1	9	F
Pediococcus ethanolidurans	J48.3	3	1	3	F
Pediococcus ethanolidurans	J48.1B	3	1	3	F
Pediococcus ethanolidurans	J48.8B	3	1	3	F
Pediococcus ethanolidurans	J48.5	3	1	3	F
Pediococcus ethanolidurans	J48.7B	3	1	3	F
Pediococcus ethanolidurans	J48.5B	3	1	3	F
Pediococcus ethanolidurans	J25.2	3	1	5	M
Pediococcus ethanolidurans	J48.10	3	1	3	F
Staphylococcus sp.	J1.12	1	1	2	I
Staphylococcus sp.	J45.12	1	1	5	F
Staphylococcus sp.	G39.31	1	2	4	M
Staphylococcus sp.	G53.5	1	2	6	F
Staphylococcus sp.	J13.4	2	1	10	I
Staphylococcus sp.	J31.12	2	1	4	M
Staphylococcus sp.	J35.13	2	1	1	M
Staphylococcus sp.	J41.1B	2	1	2	F
Staphylococcus sp.	J8.12	3	1	3	I
Staphylococcus sp.	G60.6B	4	2	5	F
Staphylococcus sp.	G57.12	5	2	2	F
Staphylococcus sp.	G57.13	5	2	2	F
Staphylococcus sp.	G55.3	5	2	8	F
Enterococcus olivae	G11.12	1	2	10	I
Enterococcus olivae	G12.11	1	2	9	I
Enterococcus olivae	G17.11	1	2	2	I
Enterococcus olivae	G19.13	1	2	4	I
Enterococcus olivae	G12.14	2	2	9	I
Enterococcus olivae	G13.14	2	2	6	I
Enterococcus olivae	G17.14	3	2	2	I
Aerococcus viridans/urinaeequi	G12.6	1	2	9	I
Aerococcus viridans/urinaeequi	G14.2	1	2	7	I
Aerococcus viridans/urinaeequi	G17.3	1	2	2	I
Aerococcus viridans/urinaeequi	G20.14	1	2	5	I
Aerococcus viridans/urinaeequi	G15.12	2	2	8	I
Aerococcus viridans/urinaeequi	G13.1	3	2	6	I
Aerococcus viridans/urinaeequi	G18.2	3	2	3	I
Aerococcus viridans/urinaeequi	G14.13	3	2	7	I
Aerococcus viridans/urinaeequi	G12.1	3	2	9	I
Aerococcus viridans/urinaeequi	G16.4	3	2	1	I
Aerococcus viridans/urinaeequi	G20.4	3	2	5	I
Aerococcus viridans/urinaeequi	G20.15	3	2	5	I
Aerococcus viridans/urinaeequi	G19.5	4	2	4	I
Aerococcus viridans/urinaeequi	G19.12	4	2	4	I
Aerococcus viridans/urinaeequi	G20.12	4	2	5	I
Aerococcus viridans/urinaeequi	G16.13	4	2	1	I
Aerococcus viridans/urinaeequi	G15.2	5	2	8	I
Aerococcus viridans/urinaeequi	G16.3	5	2	1	I
Aerococcus viridans/urinaeequi	G17.1	5	2	2	I
Aerococcus viridans/urinaeequi	G12.12	5	2	9	I
Aerococcus viridans/urinaeequi	G14.1	5	2	7	I
Aerococcus viridans/urinaeequi	G16.12	5	2	1	I
Aerococcus viridans/urinaeequi	G13.13	5	2	6	I
Aerococcus viridans/urinaeequi	G13.15	5	2	6	I
Aerococcus viridans/urinaeequi	G17.12	5	2	2	I



Aerococcus viridans/urinaeequi	G17.13	5	2	2	I
Aerococcus viridans/urinaeequi	G19.11	5	2	4	I
Aerococcus viridans/urinaeequi	G12.31	5	2	9	I
Aerococcus viridans/urinaeequi	G13.31	5	2	6	I
Aerococcus viridans/urinaeequi	G17.32	5	2	2	I
Aerococcus viridans/urinaeequi	G16.2	5	2	1	I
Aerococcus viridans/urinaeequi	G15.13	5	2	8	I
Aerococcus viridans/urinaeequi	G20.1	5	2	5	I
Aerococcus viridans/urinaeequi	G14.3	6	2	7	I
Aerococcus viridans/urinaeequi	G18.3	6	2	3	I
Aerococcus viridans/urinaeequi	G13.12	6	2	6	I
Aerococcus viridans/urinaeequi	G18.12	6	2	3	I
Aerococcus viridans/urinaeequi	G17.31	6	2	2	I
Aerococcus viridans/urinaeequi	G18.4	6	2	3	I
Aerococcus viridans/urinaeequi	G14.12	7	2	7	I
Aerococcus viridans/urinaeequi	G20.13	7	2	5	I
Aerococcus viridans/urinaeequi	G18.13	7	2	3	I
Aerococcus viridans/urinaeequi	G17.4	7	2	2	I
Aerococcus viridans/urinaeequi	G18.1	7	2	3	I
Aerococcus viridans/urinaeequi	G19.1	7	2	4	I
Aerococcus viridans/urinaeequi	G19.2	7	2	4	I
Aerococcus viridans/urinaeequi	G19.3	8	2	4	I
Aerococcus viridans/urinaeequi	G20.3	8	2	5	I
Aerococcus viridans/urinaeequi	G19.4	8	2	4	I
Aerococcus viridans/urinaeequi	G13.3	8	2	6	I
Aerococcus viridans/urinaeequi	G16.7	8	2	1	I
Aerococcus viridans/urinaeequi	G17.2	8	2	2	I
Aerococcus viridans/urinaeequi	G15.3	9	2	8	I
Aerococcus viridans/urinaeequi	G20.2	9	2	5	I
Enterococcus olivae	G15.11	4	2	8	I
Enterococcus olivae	G18.14	4	2	3	I
Enterococcus olivae	G15.1	5	2	8	I
Enterococcus olivae	G16.11^{T}	5	2	1	I
Enterococcus olivae	G18.11	5	2	3	I
Enterococcus olivae	G20.11	5	2	5	I
Pediococcus parvulus	G37.8	1	2	2	M
Pediococcus parvulus	G39.4	1	2	4	M
Pediococcus parvulus	G40.9	1	2	5	M
Pediococcus parvulus	G53.1B	1	2	6	F
Pediococcus parvulus	G53.3B	1	2	6	F
Pediococcus parvulus	G36.3	2	2	1	M
Pediococcus parvulus	G36.4	2	2	1	M
Pediococcus parvulus	G37.4	2	2	2	M
Pediococcus parvulus	G37.5	2	2	2	M
Pediococcus parvulus	G38.4	2	2	3	M
Pediococcus parvulus	G39.8	2	2	4	M
Pediococcus parvulus	G56.3	2	2	1	F
Pediococcus parvulus	G57.3	2	2	2	F
Pediococcus parvulus	G57.9	2	2	2	F
Pediococcus parvulus	G57.1B	2	2	2	F
Pediococcus parvulus	G60.1B	2	2	5	F
Pediococcus parvulus	G60.2B	2	2	5	F
Pediococcus parvulus	G34.3	3	2	7	M
Pediococcus parvulus	G34.5	3	2	7	M
Pediococcus parvulus	G34.8	3	2	7	M
Pediococcus parvulus	G34.9	3	2	7	M
Pediococcus parvulus	G54.2B	3	2	7	F
Pediococcus parvulus	G54.8	3	2	7	F
Pediococcus parvulus	J45.1	4	1	5	F
Pediococcus parvulus	J45.4	4	1	5	F
Pediococcus parvulus	J51.6	4	1	4	F
Pediococcus parvulus	J51.6B	4	1	4	F
Pediococcus parvulus	G55.9	5	2	8	F
Pediococcus parvulus	G55.1B	5	2	8	F
Pediococcus parvulus	G51.3	5	2	10	F
Pediococcus parvulus	G52.4B	5	2	9	F
Pediococcus parvulus	G55.6	5	2	8	F
Enterococcus olivae	G13.11	6	2	6	I
Enterococcus olivae	G14.11	6	2	7	I
Enterococcus olivae	G20.16	6	2	5	I
Pediococcus parvulus	G38.7	6	2	3	M
Pediococcus parvulus	G59.2B	6	2	4	F
Pediococcus parvulus	G51.4	6	2	10	F
Pediococcus parvulus	G58.6B	6	2	3	F
Pediococcus parvulus	G59.1B	6	2	4	F
Pediococcus parvulus	G39.3	7	2	4	M

Figure S2

Identification	Isolate	Genotype	Patio	Fermenter	Stage
Candida parapsilosis	J42.22	1	1	8	F
Candida glabrata	G14.22	1	2	7	I
Candida butyri/aaseri	J8.22	1	1	3	I
Candida butyri/aaseri	J8.23	1	1	3	I
Candida butyri/aaseri	J10.23	1	1	6	I
Candida butyri/aaseri	J10.22	1	1	6	I
Candida butyri/aaseri	J11.23	1	1	4	I
Candida butyri/aaseri	J1.23	2	1	2	I
Candida butyri/aaseri	J5.25	2	1	5	I
Candida butyri/aaseri	J6.22	2	1	9	I
Candida butyri/aaseri	J6.23	2	1	9	I
Saccharomyces cerevisiae	J15.22	1	1	1	I
Saccharomyces cerevisiae	J26.2	1	1	9	M
Saccharomyces cerevisiae	J21.21	1	1	2	M
Saccharomyces cerevisiae	J13.21	1	1	10	I
Saccharomyces cerevisiae	J25.21	1	1	5	M
Saccharomyces cerevisiae	J26.21	1	1	9	M
Saccharomyces cerevisiae	J28.21	1	1	3	M
Saccharomyces cerevisiae	G11.21	2	2	10	I
Saccharomyces cerevisiae	G15.21	2	2	8	I
Saccharomyces cerevisiae	G16.21	2	2	1	I
Saccharomyces cerevisiae	G17.21	2	2	2	I
Saccharomyces cerevisiae	G19.21	2	2	4	I
Saccharomyces cerevisiae	G20.21	2	2	5	I
Saccharomyces cerevisiae	G36.21	2	2	1	M
Saccharomyces cerevisiae	G12.21	2	2	9	I
Saccharomyces cerevisiae	G13.21	2	2	6	I
Saccharomyces cerevisiae	G35.21	2	2	8	M
Saccharomyces cerevisiae	J28.22	2	1	3	M
Saccharomyces cerevisiae	J31.21	2	1	4	M
Saccharomyces cerevisiae	J30.21	2	1	6	M
Saccharomyces cerevisiae	J31.7	3	1	4	M
Saccharomyces cerevisiae	J33.21	3	1	10	M
Saccharomyces cerevisiae	J34.21	3	1	7	M
Saccharomyces cerevisiae	J35.21	3	1	1	M
Saccharomyces cerevisiae	J51.21	4	1	4	F
Saccharomyces cerevisiae	J53.21	4	1	10	F
Saccharomyces cerevisiae	J55.21	4	1	1	F
Candida tropicalis	J2.23	1	1	8	I
Candida tropicalis	J5.23	1	1	5	I
Candida tropicalis	J15.23	1	1	1	I
Candida tropicalis	J8.4	1	1	3	I
Candida tropicalis	J8.9	1	1	3	I
Candida tropicalis	J5.4	1	1	5	I
Candida tropicalis	J10.24	2	1	6	I
Candida tropicalis	J5.41	3	1	5	I
Candida tropicalis	J8.41	3	1	3	I
Candida tropicalis	J1.5	3	1	2	I
Candida tropicalis	J15.13	4	1	1	I
Candida tropicalis	J1.22	4	1	2	I
Hanseniaspora sp.	J1.24	1	1	2	I
Pichia manshurica/membranifaciens	G35.22	1	2	8	M
Pichia manshurica/membranifaciens	G56.22	1	2	1	F
Pichia manshurica/membranifaciens	G58.22	2	2	3	F
Pichia manshurica/membranifaciens	G59.22	2	2	4	F
Rhodotorula mucilaginosa	J46.22	1	1	9	F
Rhodotorula mucilaginosa	J48.22	1	1	3	F
Rhodotorula mucilaginosa	J41.21	1	1	2	F
Rhodotorula mucilaginosa	J50.22	1	1	6	F
Candida thaimueangensis	J45.21	1	1	5	F
Candida thaimueangensis	J46.21	1	1	9	F
Candida thaimueangensis	J53.22	1	1	10	F
Candida thaimueangensis	J54.21	1	1	7	F
Candida thaimueangensis	J14.21	1	1	7	I
Candida thaimueangensis	G51.21	1	2	10	F
Candida thaimueangensis	G52.22	1	2	9	F
Candida thaimueangensis	G54.21	1	2	7	F
Candida thaimueangensis	G54.22	1	2	7	F
Candida thaimueangensis	G55.21	1	2	8	F
Candida thaimueangensis	G55.22	1	2	8	F
Candida thaimueangensis	G57.21	1	2	2	F
Candida thaimueangensis	G57.22	1	2	2	F
Candida thaimueangensis	G58.21	1	2	3	F
Candida thaimueangensis	G32.21	1	2	9	M
Candida thaimueangensis	G37.21	1	2	2	M
Candida thaimueangensis	J42.21	2	1	8	F
Candida thaimueangensis	J48.21	2	1	3	F
Candida thaimueangensis	J50.21	2	1	6	F
Candida thaimueangensis	G51.22	3	2	10	F
Candida thaimueangensis	G52.21	3	2	9	F
Candida thaimueangensis	G60.21	4	2	5	F
Candida thaimueangensis	G60.22	4	2	5	F
Candida thaimueangensis	G56.21	4	2	1	F
Candida thaimueangensis	G53.21	5	2	6	F
Candida thaimueangensis	J22.22	6	1	8	M
Candida thaimueangensis	J26.22	6	1	9	M
Candida thaimueangensis	J33.22	6	1	10	M
Candida thaimueangensis	J34.22	6	1	7	M
Candida thaimueangensis	G53.22	6	2	6	F
Candida thaimueangensis	G59.21	7	2	4	F
Issatchenkia orientalis	J5.21	1	1	5	I
Issatchenkia orientalis	J15.21	1	1	1	I
Issatchenkia orientalis	J11.21	1	1	4	I
Issatchenkia orientalis	J1.3	1	1	2	I
Issatchenkia orientalis	J2.5	1	1	8	I
Issatchenkia orientalis	J5.5	1	1	5	I
Issatchenkia orientalis	J8.2	1	1	3	I
Issatchenkia orientalis	J1.21	1	1	2	I
Issatchenkia orientalis	J2.21	1	1	8	I
Issatchenkia orientalis	J10.21	1	1	6	I
Issatchenkia orientalis	J10.9	1	1	6	I
Issatchenkia orientalis	J11.4	1	1	4	I
Issatchenkia orientalis	J1.42	1	1	2	I
Issatchenkia orientalis	J11.41	1	1	4	I
Issatchenkia orientalis	J6.21	1	1	9	I
Issatchenkia orientalis	J2.42	2	1	8	I
Issatchenkia orientalis	J8.42	2	1	3	I
Kluyveromyces lactis/marxianus	G38.21	1	2	3	M
Kluyveromyces lactis/marxianus	G39.21	1	2	4	M
Kluyveromyces lactis/marxianus	G40.21	1	2	5	M
Kluyveromyces lactis/marxianus	G31.21	1	2	10	M
Hanseniaspora sp.	J13.22	2	1	10	I
Hanseniaspora sp.	G11.22	2	2	10	I
Hanseniaspora sp.	J6.24	2	1	9	I
Saturnispora mendoncae	J2.24	1	1	8	I
Saturnispora mendoncae	J5.24	1	1	5	I
Saturnispora mendoncae	J8.24	1	1	3	I

Figure S3

4.5 PCR-DGGE assessment of the bacterial diversity in Spanish-style green table-olive fermentations.

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PCR-DGGE assessment of the bacterial diversity in Spanish-style green table-olive fermentations

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ABSTRACT

The bacterial ecology associated to Spanish-style green olive fermentations has been studied, attending to its dynamics along the time and its distribution, by a culture-independent approach based on PCR-DGGE. Forty-three 10-tonne fermenters were selected from the fermentation yards (*patios*) of two large table-olive manufacturing companies in southern Spain. The fermenting brines of 20 of these fermenters were previously analyzed through culture-dependent methods, allowing comparisons of both methodologies. A statistical analysis of DGGE banding profiles obtained using bacteria universal primers demonstrated significant evidences of discrimination of bacterial communities by location (*patio*) and fermentation stage. Specific microbial "fingerprints" could be established for these variables. At least 17 bacterial species were detected, most of them previously isolated from the same fermenters. Most of these species belonged to the lactic acid bacteria (LAB) group. Dominance of species within the *Lactobacillus plantarum* group was confirmed. *Marinilactibacillus* sp. and *Propionibacterium olivae*, which were not isolated in the previous culture-dependent study, were detected. *Alkalibacterium* sp. and *Halolactibacillus* sp. were detected for the first time in table olive fermentations. Using *Lactobacillus*-group specific primers, significant clustering within the DGGE banding profiles was observed, allowing discrimination regarding the actual fermentation stage. These results corroborated the previous culture-dependent study, and added the detection of *Alkalibacterium* sp. and *Pediococcus acidilactici*. The species *Alkalibacterium* sp., *Marinilactibacillus* sp. and *Halolactibacillus* sp. are characterized by their ability to carry out lactic acid fermentation under alkaline conditions and thus ascribed within the halophilic and alkaliphilic lactic acid bacteria (HALAB). Their ubiquitous presence suggests that they could play an important role in Spanish-style olive fermentations, especially at the initial fermentation stage. Thus, they could contribute to brine conditioning before *L. plantarum* group-driven lactic acid fermentation takes place.

1. Introduction

Spanish-style green table olives, a fermented food of major economic importance in Mediterranean countries, are elaborated following traditional methods where spontaneous lactic acid fermentation takes place. This fermentation is usually driven by the microbiota acquired during the processing of fruits at the factory's facilities and fermentation yards (*patios*). Therefore, like for other vegetable fermentations, organoleptic characteristics as well as preservation properties are very dependant on the autochthonous microbiota which is present in its processing environment (Giraffa, 2004; Ruiz-Barba and Jiménez-Díaz, 2012). Natural fermentations are usually characterized by a diverse and complex microbiota which may be difficult to examine using conventional microbiological, i.e. culture-dependent, methods (Ampe et al., 1999; Giraffa, 2004; Justé et al., 2008). The need for microorganism cultivation prior to identification through biochemical or molecular methodology has limited the knowledge of the actual microbial diversity. Thus, it has been postulated that above 90% of the microorganisms present in natural environments cannot be cultivated through conventional microbiological techniques (Amann and Kuhl, 1998). Hugenholtz et al. (1998) raised this figure to 99%. Actually, it has been estimated that at least 25–50% of the active microbial community of fermented foods cannot be cultivated in the laboratories (Ampe et al., 1999). To surpass these limitations, culture-independent techniques appeared in the 90s as different applications of the recently discovered PCR (Mullis et al., 1986). The technique that has most often been used as a culture-independent method to study microbial communities in food

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microbiology is PCR combined with denaturing gradient gel electrophoresis (PCR-DGGE) (Cocolin et al., 2013a). Recently, PCR-DGGE has been successfully applied by several authors to study table olive fermentations (Abriouel et al., 2011; Cocolin et al., 2013b; Muccilli et al., 2011; Randazzo et al., 2012). Typically, this technique involves the extraction of nucleic acids from the fermenting brines, followed by PCR amplification of 16S rDNA gene fragments, using universal or group-specific primers, and separation of the resulting PCR products by DGGE. The banding pattern of a sample can then be compared to that of known microbial species (included in ad hoc DGGE markers) or other microbial community profiles. In addition, fragments of interest (usually those with no matching to the markers) may also be excised from the gels, reamplified through PCR, sequenced, and compared to 16S rDNA databases for identification.

Recently, our group has reported comprehensive studies on the microbiota which is associated to Spanish-style green table-olive fermentations, both at the species and strain levels (Lucena-Padrós et al., 2014b,d). In these studies, more than one thousand microbial isolates were identified, which belonged to 49 bacterial and yeast species, included in 25 different genera. In addition, a novel bacterial species was isolated and described, i.e. *Enterococcus olivae* sp. nov. (Lucena-Padrós et al., 2014a). Therefore, a high biodiversity could be observed in this food fermentation through culture-dependent techniques. However, according to the abovementioned observations of environmental microbiologists, a large proportion of microorganisms that are difficult or virtually impossible to detect with these techniques may have been excluded for different reasons (Giraffa and Neviani, 2001; Hugenholtz et al., 1998). Therefore, these previous studies may not reflect the complete, real microbial communities involved in this fermentation. Consequently, a polyphasic approach involving culture-independent techniques should be adopted to attempt a more realistic view of the composition of this microbial community and its dynamics over fermentation time.

The aim of the present study is to assess the bacterial diversity of Spanish-style green olive fermentations through a culture-independent technique such as PCR-DGGE.

2. Materials and methods

2.1. Origin of the samples and sampling strategy

Samples of Spanish-style green-olive fermenting brines were obtained from 43 10-tonne fermenters at two large manufacturing companies in the province of Seville, southern Spain. Samples were taken along the fermentation time (ca. 3 months) in coincidence with the initial, middle and final characteristic stages of green olive fermentations during the season 2010–2011. More specifically, fermentation had taken place for 1 to 14 (first two weeks), 35 to 48 (5th to 7th week), and 69 to 72 (10th to 12th week) days after brining, for the initial, middle and final sampling points, respectively. Twenty of these fermenting brines, 10 of them at each company (numbered consecutively 1–10 in this work to be consistent with previous publications), have been analyzed previously through culture-dependent techniques (Lucena-Padrós et al., 2014b,d). Fermentation set up as well as sampling strategy and sampling times have been previously described for these fermenters (Lucena-Padrós et al., 2014b) and are valid also for the rest of the fermenters analyzed in this study. Fifty ml samples were taken from the geometric centers of the fermenters as previously described (Lucena-Padrós et al., 2014b). Within *patio* 1, two different areas (1a and 1b, including 15 and 5 fermenters, respectively) were considered, while three different areas (2a, 2b and 2c, including 12, 8 and 3 fermenters, respectively) were considered in *patio* 2. Areas were defined considering their construction date and location within the *patio*, as well as the fact of sharing common facilities and equipment. Samples were stored at −80 °C in 20% (v/v) glycerol until analyzed.

2.2. DNA extraction from brine samples

Extraction of genomic DNA from brine samples was performed according to a modification of the protocol described by Martín-Platero et al. (2007) as follows: 3 ml of brine per sample was subjected to centrifugation at 13,000 rpm at 4 °C for 60 s and the resulting pellet was washed with 1 ml of TESAC buffer (10% sucrose, 25 mM Tris–HCl [pH 8.0], 10 mM EDTA). After a new centrifugation, the supernatant was discarded while the pellet, containing microbial cells, was resuspended in 100 μl of TESAC buffer supplemented with 10 mg/ml of freshly-made lysozyme (Sigma, St. Louis, MO) plus 40 μg/ml RNase (Sigma) and incubated at 37 °C for 30 min. The resulting protoplast cells were immediately lysed by adding 600 μl of lysis buffer (100 mM Tris–HCl [pH 8.0], 100 mM EDTA, 10 mM NaCl, 1% SDS) with the tube being gently inverted to mix thoroughly. The mixture was then incubated at room temperature for 15 min. To increase the purity of the DNA, the lysate was treated with 10 μl of proteinase K (10 mg/ml, Sigma) and incubated at 37 °C for 15 min. In addition, incubation at 55 °C for 15 min followed by cooling to room temperature for 10 min was included to improve cell lysis and DNA yields. Subsequently, 200 μl of cold 7.5 M ammonium acetate was added, the solution was vortex-mixed for 10 s, chilled on ice for 10 min and then centrifuged at 13,000 rpm for 10 min to precipitate the proteins. Finally, the nucleic acids present in the supernatant were precipitated with an equal volume of isopropanol (ca. 950 μl) in a clean microfuge tube, which was gently inverted several times and chilled on ice for 10 min. The genomic DNA was pelleted by centrifugation at 13,000 rpm for 10 min., washed once with 1 ml of 70% (v/v) ethanol and dried at room temperature. The washed DNA pellet was finally resuspended in 200 μl of molecular grade deionized water (Sigma). Samples (5 μl) were then analyzed by agarose gel electrophoresis to probe their integrity and estimate their concentration.

2.3. PCR amplification and DGGE analysis

To investigate the dominant bacterial communities by DGGE analysis, PCR products were generated using universal primers U968-GCf and L1401r that amplify the V6 to V8 region of bacterial 16S rDNA (Nubel et al., 1996). The 40-nucleotide GC rich sequence at the 5′ end of primer U968-GCf improves the detection of sequence variations of amplified DNA fragments by subsequent DGGE running (Muyzer et al., 1993). PCR amplification was performed as previously described (Martín et al., 2007a). *Lactobacillus* group-specific PCR was performed using primers targeting the V2–V3 region of the bacterial 16S rDNA gene. To prevent a low amplicon yield, a nested PCR approach was used as described earlier (Heilig et al., 2002). This involved a first PCR reaction in which primers Bact27f (Lane, 1991) and Lab-677r (Heilig et al., 2002) were used, followed by a second PCR with primers Lab159f (Heilig et al., 2002) and Univ515-GCr (Lane, 1991). A 40-bp GC-clamp was attached to the 5′ end of the Uni515r primer in order to facilitate the analysis of the PCR products by DGGE. PCR was performed as previously described (Martín et al., 2007b). PCR products that were used as templates in nested PCR were purified with the NucleoSpin Extract II (Macherey-Nagel, Düren, Germany). PCR products were stored at −20 °C until further use.

DGGE analysis of PCR amplicons was performed on the Dcode System apparatus (BioRad), as previously described (Muyzer et al., 1993). Samples were loaded into an 8% (w/v) polyacrylamide gel (acrylamide:bisacrylamide 37.5: 1) in 0.5 TAE buffer (2 M Tris base, 1 M glacial acetic acid, 50 M EDTA pH 8.0). Optimal separation of the PCR products obtained with bacterial 16S rDNA primers was achieved with 30–60% urea formamide denaturant gradient, increasing in the direction of electrophoresis, while optimal separation of the *Lactobacillus* group-specific PCR products was achieved with 35–55% urea formamide denaturant gradient, increasing in the direction of electrophoresis. A 100% denaturant corresponds to 7 M urea and 40% (v/v) formamide.

Electrophoresis was performed at a constant voltage of 75 V and at temperature of 60 °C for 16 h. The DNA bands were visualized by silver staining as previously described (Sanguinetti et al., 1994). It was possible to digitally normalize the DGGE profiles by comparison with a standard pattern (DGGE marker) using BioNumerics 7.0 software package (Applied Maths, Sint-Martens-Latem, Belgium). This normalization enabled comparison between DGGE profiles from different gels that were run under comparable denaturing and eletrophoretic conditions. Cluster analysis of DGGE pattern profiles was performed using the UPGMA method based on the Dice's similarity coefficient (band based).

2.4. Statistical analysis of bacterial community structure

Similarities between microbial community profiles generated by DGGE analysis were assessed by Dice similarity index and the UPGMA clustering algorithm using the BioNumerics 7.0 software package. The same software package was employed in the discriminant analysis of bacterial community banding patterns that was performed by the jackknife method to evaluate the stability of the resulting clustering and the integrity of the assignments of banding patterns to defined groups based on fermentation stage, *patio* or area within each *patio*. The percentage of correctly assigned observations for all fingerprints was computed and reported as the estimated rate of correct classification (ERCC) calculated as proposed by Ringbauer et al. (2006). The random ERCC was conservatively calculated as described in James et al. (2006). Expected values for a null hypothesis of random association were computed by comparing the percentages expected under the null hypothesis with the confidence limits for the percentage correctly classified. Confidence limits for the percentage correctly classified were obtained from statistical tables (Sokal and Rohlf, 1987). Moreover, the similarity matrix calculated from the presence–absence data sets using the Dice's coefficient was used as data for the multidimensional scaling map (MDS) method, in which data were represented in a Euclidean plane (van Hannen et al., 1999; Bernhard et al., 2005). Every band pattern was shown as one plot, and highly similar band patterns were plotted close together. MDS analysis was performed using SPSS 21.0 software package (SPSS Limited-IBM, Woking, UK). DGGE fingerprintings obtained with bacteria universal primers were further analyzed in terms of the phylotype richness (S; number of bands) (Sigler et al., 2004). U Mann–Whitney tests were applied to determine statistically significant differences between the S-values in both *patios* at each fermentation stage whereas the fermentation–time effect on phylotype richness in each *patio* was tested using Friedman tests. Significances were accepted at a level of probability (p) of 0.05.

2.5. Marker lanes

In order to enable the identification of bands in DGGE fingerprints, specific markers were built. Total DNA from pure cultures of a variety of selected bacterial species was obtained from isolated colonies using the rapid chloroform method described by Ruiz-Barba et al. (2005). Subsequent PCR was carried out applying the same procedure described above for brine samples. The resulting amplicons were purified using the NucleoSpin Extract II kit and equilmolar amounts were mixed to obtain the DGGE markers. Three markers were used in DGGE analyses with universal primers U968-GCf and L1401r. Marker I comprised amplicons from (in increasing electrophoretic migration order) *Staphylococcus* sp. (epidermidis group) J1.12, *Clostridium xylanovorans* J6.31, *Clostridium jejuense* J14.32, *Clostridium schirmacherense*/*argentinense* J8.31, *Clostridium sporogenes* C22/10, and *Clostridium tyrobutyricum* 3.5 (Fig. S1). Marker II comprised amplicons from *Lactobacillus pentosus* G11.2, *Lactobacillus parafarraginis* J31.6, *Lactobacillus collinoides*/*paracollinoides* J30.1, *Pediococcus parvulus* G72.8, *Lactobacillus paracasei* G31.12, *Pediococcus ethanolidurans* G73.4, and *Lactobacillus coryniformis* G38.8 (Fig. S1). Marker III comprised amplicons from *Aerococcus viridans*/*urinaeequi* G18.4, *Enterococcus casseliflavus* J1.9, *Weissella paramesenteroides*/*hellenica* J11.1, *Enterococcus faecium* J19.2, *Enterococcus faecalis* J20.4, *E. olivae* IGG16.11^T, *Propionibacterium olivae* IGBL1^T and *Propionibacterium acnes* J41.31. Marker IV was used in DGGE analyses of lactobacilli and related lactic acid bacteria (*Lactobacillus* group) with specific PCR primers, and comprised amplicons from *Lactococcus lactis* ssp. *lactis* IL1403, *Leuconostoc mesenteroides* L32, *P. parvulus* G72.8, *Pediococcus acidilactici* 347, *Lactobacillus rapi* G51.2B, *L. collinoides*/*paracollinoides* J30.1, *L. parafarraginis* J31.6, *Lactobacillus rhamnosus* G32.8, and *L. paracasei* G31.12 (Fig. S1). Strains used in these reference markers are natural isolates from Spanish-style green olive fermenting brines that were obtained and identified in a previous biodiversity analysis by culture-dependent methods in the same *patios* (Lucena-Padrós et al., 2014d) or belong to our collection and are representative of some of the bacterial species that might be associated to table olive fermentations.

2.6. Identification of DGGE electrophoretic bands

The bands observed in the DGGE analyses were identified using two different approaches. The first approach consisted on the comparison of the DGGE profiles of the samples with DGGE markers constructed previously (Fig. S1). In addition, notorious DGGE bands that could not be identified by comparison with the DGGE markers were excised from the gels with sterile surgical blades and DNA was extracted using the protocol of Sanguinetti et al. (1994). Extracted DNA was used as a template for PCR reamplification using the primer pairs U968f (devoid of the GCclamp)/L1401r or Lab159f/Univ515r (devoid of the GCclamp) for DNA products which were primarily obtained from PCRs with universal or *Lactobacillus*-group specific primers, respectively. The resulting PCR products were purified using the NucleoSpin Extract II kit and sequenced. The sequences obtained were compared to those of type strains present in the NCBI database using the BLAST algorithm and their identity was determined based on the highest scores.

3. Results

3.1. Distribution of the bacteria community through PCR-DGGE analysis

PCR-DGGE banding patterns obtained with bacteria universal primers showed that amplicons were concentrated along the middle-bottom side of the gel gradient, i.e. in the range 45–60% urea. These banding patterns, organized in a dendrogram as a result of a UPGMA clustering analysis, are shown in Fig. S2. An example of the evolution of banding patterns in 8 fermenters along the fermentation time is also shown in Fig. 1. Overall, profiles from *patio* 1 were significantly less complex ($p < 0.05$) in terms of band number (phylotype richness) than *patio* 2 ones (Fig. 2). On the other hand, considering the dynamics of bacterial populations along the time, significant differences in phylotype richness could be found only in *patio* 2 (Fig. 2). Cluster analysis of DGGE profiles through the UPGMA method is shown in Fig. S2. A discriminant analysis of this clustering was carried out by the jackknife method. This analysis revealed that assignments of samples to specific groups based on location (*patio*) provided a consistent effect in the bacterial community composition ($p < 0.05$). This is shown in Table 1, where an ERCC value of 72.22% was obtained whereas the random ERCC was 52.38%. It was remarkable that incorrect assignment of samples was observed only in samples from *patio* 1 (Table S1). This result is graphically visualized in the MDS plot shown in Fig. 3, where samples from *patio* 1 showed a dispersed distribution while configuration of samples from *patio* 2 indicated the presence of a central core data group. Dimensions (axes x and y) of the spatial configuration map, generated by MDS scores, are simply for plotting purposes, although distances between data points indeed reflect the relationships between samples in the underlying data set. Furthermore, samples also grouped regarding the fermentation stage significantly ($p < 0.05$) (Tables 1 and

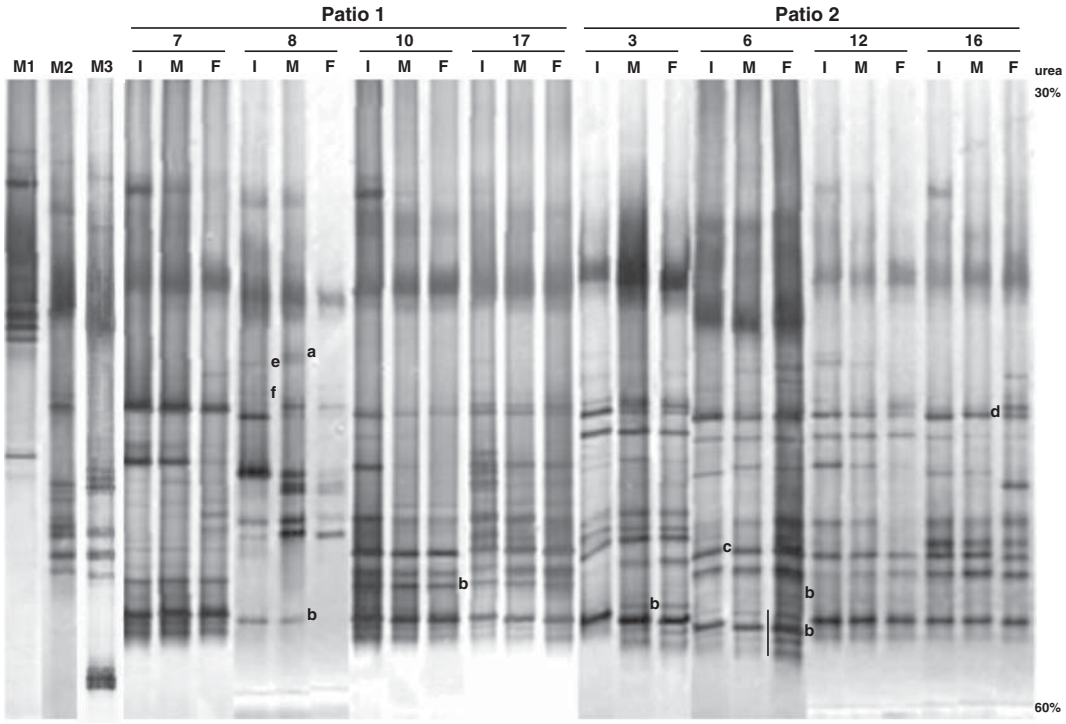

Fig. 1. Normalized PCR-DGGE profiles of 16S rRNA gene V6–V8 variable regions, representing the biodiversity of bacteria in the fermentation brines of eight fermenters from two different fermentation yards (*patios*) at each fermentation stage (I, initial; M, middle; F, final). M1, M2 and M3, PCR-DGGE identification markers used in this study. The classes of bands identified by gel excision and subsequent DNA sequencing are indicated at the right side of each band as follows: a, *Lactobacillus collinoides/paracollinoides*; b, *Alkalibacterium* sp. (the black bar indicates a group of four characteristic bands for species within this genus); c, *Marinilactibacillus* sp.; d, *Halolactibacillus* sp.; e, *Vibrio* sp.; and f, *Vibrio furnissii/fluvialis*.

S2). As it was expected, analysis of the frequency of incorrect assignments at each fermentation stage revealed that changes in bacterial community structure were gradual, being this composition more similar at the middle and final fermentation stages. Dynamics of bacterial communities in each fermenter along the fermentation time is also reflected in the MDS plot shown in Fig. 3, where mean changes in bacterial community structure (distance between data points) were higher between samples from the initial to the middle fermentation stages than from the middle to the final stages in most cases. When samples were grouped combining multiple significant factors such as location (*patio*) and fermentation stage, this resulted in a statistically significant ($p < 0.05$) partitioning of the samples, with an ERCC value of 56.35% while the random ERCC was 18.25 (Table 1). Only 4.76% of the samples

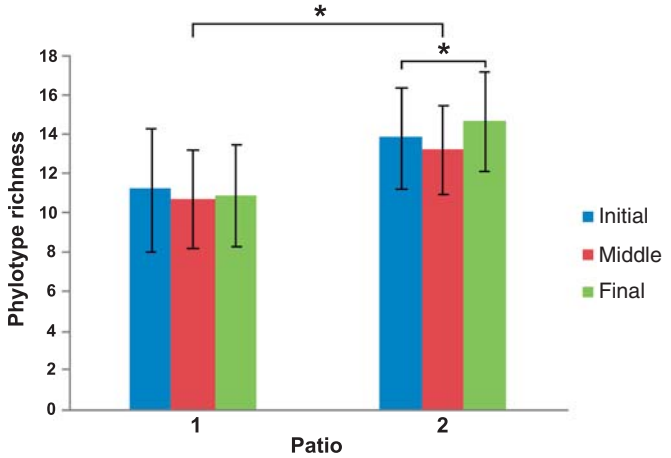

Fig. 2. Phylotype richness of DGGE profiles at each fermentation stage in two Spanish-style table-olive fermentation yards (*patios*). Richness data are shown as mean number of bands on the DGGE profiles with SEM. *Statistically significant difference ($p < 0.05$).

(6 cases out of 126) sorted incorrectly when considering both mentioned factors (Table S3). Finally, when a discriminant analysis considering the different areas within each location (*patio*) was performed, significant differences were found only in the area sub-set from *patio* 2 (Table 1).

3.2. Identification of the bacteria community through PCR-DGGE

To identify the bands observed in the PCR-DGGEs, DGGE profiles of the samples (Figs. 1 and S2) were compared to those of the three different markers constructed ad hoc (markers I, II and III in Fig. S1). Matching banding patterns were found for all of the species in marker II, except for *L. coryniformis*, and marker III, except for *E. faecalis* and *E. faecium* (Table 2). However, none of the species included in marker I were detected. When DGGE bands which did not match any of those species included in the markers were excised from the gels, purified and sequenced, the presence of *Alkalibacterium* sp., *Halolactibacillus* sp., *Marinilactibacillus* sp., *Vibrio furnissii/fluvialis*, and *Vibrio* sp. was revealed (Table 2, Figs. 1 and S2). Also, extra bands corresponding to *L. collinoides/paracollinoides* were detected (Figs. 1 and S2).

Table 1
Comparisons of ERCCs of different groupings made after PCR-DGGE analysis of the bacterial community in Spanish-style olive fermentations by jackknife analysis (Dice's coefficient).

Group type	ERCC (%)	Random ERCC (%)	Statistical difference ($p < 0.05$)
Location (*patio*)	72.22	52.38	Yes
Fermentation stage	61.90	34.13	Yes
Location × fermentation stage	56.35	18.25	Yes
Area			
Sub-set: *patio* 1	83.33	75	No
Sub-set: *patio* 2	68.20	54.55	Yes

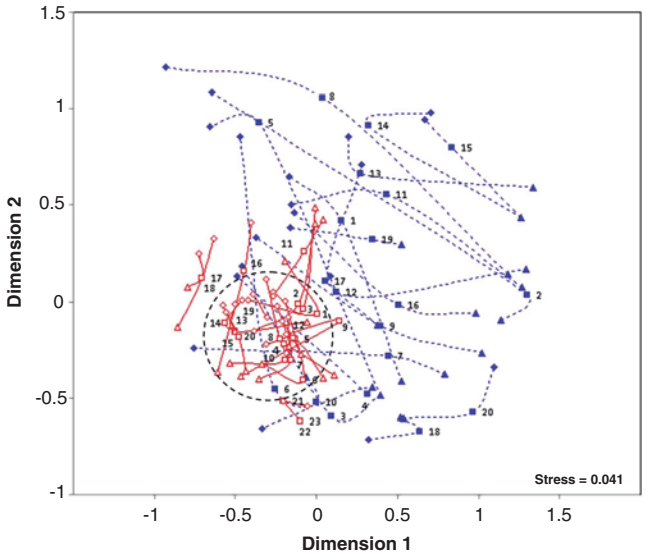

Fig. 3. Multidimensional scaling ordinations of pairwise estimates of similarity (Dice's coefficient) calculated from PCR-DGGE profiles using bacteria universal primers during Spanish-style green olive fermentations in two fermentation yards (*patios*). Data at each fermentation stage considered (triangles, initial; squares, middle; rhombus, final) of the same fermenter are connected by lines (dot lines and solid symbols, fermenters from *patio* 1; lines and open symbols, fermenters from *patio* 2). Core data group of *patio* 2 fermenters is circled. Value of normalized stress is indicated.

3.3. Distribution of the Lactobacillus-group community through PCR-DGGE analysis

PCR-DGGE profiles using *Lactobacillus*-group specific primers were obtained from samples of fermenting brines at *patio* 2. These DGGE profiles are shown in Fig. S3, where they are organized in a dendrogram as a result of a UPGMA clustering analysis. Such clustering shows the dynamics of the *Lactobacillus*-group community across the fermentation time. This was corroborated by a discriminant analysis of banding-pattern assignment performed by the jackknife method, showing that the mean of ERCC regarding the fermentation stage was significantly higher than the calculated random ERCC (Table 3). However, the mean of ERCC regarding the different areas within *patio* 2 was significantly lower than the random ERCC value (Table S4). Therefore, PCR-DGGE profiles of samples collected from fermenters placed in different areas within *patio* 2 did not show a clear discrimination regarding the *Lactobacillus*-group community.

Table 2
Prevalence of bacterial species identified through PCR-DGGE using universal primers at each fermentation stage (initial, middle and final) of Spanish-style green olive fermentations in two different fermentation yards (*patios*).

Species	Patio 1			Patio 2		
	Initial	Middle	Final	Initial	Middle	Final
Aerococcus viridans/urinaeequi	10.0[a]	10.0	10.0	95.0	95.7	100
Alkalibacterium sp.[b–d]	100	100	95.0	100	100	100
Enterococcus casseliflavus	95.0	80.0	55.0	55.0	60.9	52.2
Enterococcus olivae	55.0	50.0	50.0	80.0	100	100
Halolactibacillus sp.[b–d]	0.0	0.0	0.0	50.0	4.4	4.4
Lactobacillus paracasei/rhamnosus	55.0	65.0	15.0	40.0	39.1	47.8
Lactobacillus parafarraginis	10.0	35.0	65.0	0.0	13.0	43.5
Lactobacillus collinoides/paracollinoides	15.0	55.0	85.0	100	78.3	87.0
Lactobacillus plantarum group[e]	100	100	100	100	100	100
Marinilactibacillus sp.[b,c,d]	50.0	60.0	60.0	100	100	100
Pediococcus ethanolidurans	10.0	20.0	25.0	0.0	0.0	0.0
Pediococcus parvulus	15.0	20.0	60.0	100	95.7	95.7
Propionibacterium olivae[c]	10.0	0.0	10.0	0.0	0.0	0.0
Propionibacterium acnes	10.0	0.0	10.0	0.0	0.0	0.0
Vibrio furnissii/fluvialis[b]	85.0	0.0	0.0	0.0	0.0	0.0
Vibrio sp.[b]	85.0	5.0	0.0	30.0	4.4	0.0
Weissella paramesenteroides/hellenica	30.0	35.0	30.0	65.0	52.2	56.5

[a] Results are expressed as percentage of fermenters where a specific bacterial species was detected through PCR-DGGE.
[b] Bacterial species which were detected after extraction of the corresponding PCR-DGGE band and subsequent DNA sequencing.
[c] Bacterial species which were not detected previously in the fermenters under study through culture-dependent techniques (Lucena-Padrós et al., 2014b).
[d] Bacterial species which have not been previously isolated from table olive fermentations.
[e] Includes the species *Lactobacillus plantarum*, *Lactobacillus pentosus* and *Lactobacillus paraplantarum*, which were indistinguishable using this technique.

Table 3
Assignment of banding patterns to fermentation-stage groups of the *Lactobacillus*-group community in Spanish-style olive fermentations by jackknife analysis (Dice's coefficient).

Fermentation stage	Banding patterns (%) assigned to groups		
	Initial (n = 20)	Middle (n = 23)	Final (n = 23)
Initial	**85.0**	4.3	0.0
Middle	5.0	**60.9**	0.0
Final	10.0	34.8	**100**

ERCCs of patterns to host class are in boldface. The mean ERCC was 81.82%, while the random ERCC was 34.85%. Differences are statistically significant ($p < 0.05$).

3.4. Identification of the Lactobacillus-group community through PCR-DGGE

Comparison of *Lactobacillus* group-specific PCR-DGGE profiles to marker IV allowed the identification of all the species contained in this marker but *L. lactis* and *L. mesenteroides* (Table 4). These two species had not been isolated from these fermenters in a previous culture-dependent study either (Lucena-Padrós et al., 2014b). However, the presence of *P. acidilactici*, which had not been isolated previously, was observed and verified after band extraction and DNA sequencing (Table 4). When DGGE bands which did not match any of the species included in marker IV were excised from the gels, purified and sequenced, the presence of *A. viridans*, *Alkalibacterium* sp., and species of the *Lactobacillus plantarum* group was also revealed (Table 4). The relative presence of the bacterial species detected in this way throughout the fermentation time is also shown in Table 4.

Table 4
Prevalence of bacterial species identified through PCR-DGGE using *Lactobacillus*-group specific primers at each fermentation stage (initial, middle and final) of Spanish-style green olive fermentations in fermentation yard (*patio*) #2.

Species	Fermentation stage		
	Initial	Middle	Final
Aerococcus viridans[a]	95.0[b]	26.1	39.1
Alkalibacterium sp.[a,c,d]	25.0	13.0	8.7
Lactobacillus paracasei	50.0	78.3	60.9
Lactobacillus parafarraginis	5.0	82.6	91.3
Lactobacillus collinoides/paracollinoides	20.0	73.9	95.7
Lactobacillus plantarum group[a,e]	100	95.7	95.7
Lactobacillus rapi	25.0	8.7	26.1
Lactobacillus rhamnosus	0.0	47.8	30.4
Pediococcus acidilactici[c]	5.0	4.4	8.7
Pediococcus parvulus	0.0	26.1	65.2

[a] Bacterial species which were detected after extraction of the corresponding PCR-DGGE band and subsequent DNA sequencing.
[b] Results are expressed as percentage of fermenters where a specific bacterial species was detected through PCR-DGGE.
[c] Bacterial species which were not detected previously in the fermenters under study through culture-dependent techniques (Lucena-Padrós et al., 2014b).
[d] Bacterial species which have not been previously isolated from table olive fermentations.
[e] Species within the *L. plantarum* group, including *L. plantarum*, *L. pentosus* and *L. paraplantarum*, are not distinguishable by 16S rDNA sequence analysis.

4. Discussion

To our knowledge, this is the first study on bacterial community diversity and its dynamics made on industrial-scale traditional Spanish-style green table-olive fermentations carried out using a culture-independent approach such as PCR-DGGE. As near 50% of the fermenters had been analyzed through culture-dependent techniques in a previous study (Lucena-Padrós et al., 2014b), these results allow a quite relevant comparison of both methodologies.

Clustering analysis of the PCR-DGGE profiles obtained with bacteria universal primers showed a trend of grouping by location (*patio*), which was corroborated by jackknife analysis of similarity coefficients (Table 1). As expected, these results confirmed that the actual *patio* was a statistically significant factor influencing the bacterial community structure. Therefore, PCR-DGGE analysis could be a powerful tool for scientific purposes or even control authorities to characterize fermenting-olive brine samples and ascribe them to a specific location, contributing so to the traceability of the product. It could also allow the assessment of whether a normal fermentation is taking place in the fermenters within a specific *patio* or some alteration, which could end up in spoilage of the product, is taking place. Corrective steps could be done in time in the last case. Clustering and jackknife analyses performed on the PCR-DGGE profiles showed that the fermentation stage factor exerts also a quite significant effect on bacterial community structure (Table 1). Through this methodology it could be determined whether a brine sample is naturally evolving through the fermentation time. Early detection of spoilage microorganisms would be very helpful especially at the final fermentation stage. Actually, *P. olivae* and *P. acnes* could be detected, two microorganisms which can cause spoilage at the end of the usual lactic acid fermentation of table olives, in 10% of the fermenters at *patio* 1 (Table 2). In fact, *P. olivae* was first isolated, and described as a novel species, from spoiled packaged Spanish-style green table olives (Lucena-Padrós et al., 2014c). On the other hand, the discriminant analysis by jackknife method revealed significant differences between PCR-DGGE profiles among the areas considered within *patio* 2. This makes sense, for the two areas defined within *patio* 1 are the same age, while those within *patio* 2 were built and utilized for the first time at quite different seasons, as declared by the respective manufacturing companies' personnel. The facilities and equipment used for the initial processing of the raw fruits, i.e. the characteristic alkali treatment and subsequent washing of the excess of lye, are unique in both *patios*. Therefore, differences in the microbiota should be due mainly to the fermenters themselves, especially to the number of seasons they have been used previously as well as their specific location within each *patio*. A microbial "fingerprint" which is characteristic of a specific *patio* could be determined, as it was pointed out in a recent publication (Lucena-Padrós et al., 2014d).

Identification using universal primers revealed the presence of at least 17 different bacterial species (Table 2). Most of these species belonged to the LAB group. Although most unknown electrophoretic bands were extracted and prepared for DNA sequencing, not all of them rendered a result of enough quality as to be considered. This fact could be due to the use of silver staining of the DGGE gels that, although offering more sensitivity than other staining methods, it is known to cause trouble in subsequent PCR amplifications and sequencing (as well as more background problems). At least 13 of the species identified with this procedure had been already detected through culture-dependent techniques applied on 20 of the fermenters studied here, when up to 37 different species were isolated and identified (Lucena-Padrós et al., 2014b). On the other hand, some species were not isolated in the cited previous study. This is the case of *Marinilactibacillus* sp., *P. olivae*, *Alkalibacterium* sp. and *Halolactibacillus* sp. Actually, the last two species had not been described before in table olives. However, some of the species included in the markers were not detected in any of the sample. This is the case of those included in marker I, which included species which could be involved in table olive spoilage, and also *L. coryniformis* and *E. faecium/faecalis*, in markers II and III, respectively. In total, up to 24 bacterial species which were isolated in the previous culture-dependent study were not detected now. Some authors have postulated that the detection of individual members from mixed microbial populations cannot be detected by PCR-DGGE when their concentration is lower than 10^3 CFU/ml or g (Cocolin et al., 2001a,b). Actually, increasing complexity of these populations can raise this detection limit according to these authors. In the present case, only *Staphylococcus* sp. and *L. coryniformis*, which were previously isolated from these samples in the range 10^1–10^5 and 10^4–10^6 CFU/ml, respectively, should have been detected according to the mentioned criterion. In the case of *L. rapi*, previously isolated in the range 10^3–10^6 CFU/ml, was actually detected through PCR-DGGE using *Lactobacillus* group-specific primers (Table 4). The elevated complexity, in terms of microbial species, determined through culture-dependent techniques in these table-olive fermenting brines could explain the failure in the detection of some species. This fact could be alleviated by the use of group-specific primers, which enhances the sensitivity of this technique by reducing the complexity of the population under study.

In *patio* 2, the distribution of the *Lactobacillus*-group community by using specific primers was studied. This appeared an obvious selection as it was previously determined, by culture-dependent techniques, that a LAB such as *L. pentosus* dominated these fermentations and more than 43% of the bacterial species isolated belonged to the LAB group (Lucena-Padrós et al., 2014b). Clustering of the corresponding PCR-DGGE profiles showed that they could be grouped based on the fermentation stage at which the samples were taken. This fact could be quite helpful to determine if a correct fermentation is taking place without the use of time-consuming traditional techniques. However, discrimination by areas was not possible, perhaps indicating that the *Lactobacillus*-group community is well installed and adapted in all of the fermenters in this *patio*, regardless of their age. Although it was known that *L. pentosus* was the dominant species in these fermentations, it was not included in the corresponding marker because of the existence of a characteristic "fuzzy" band appearing when the primers used in this study were applied to the species constituting the *L. plantarum* group (Fig. S3). Martín et al. (2007b), when studying the diversity of the *Lactobacillus* group in breast milk and vagina through PCR-DGGE, reported the same observation for *L. plantarum*. Electrophoretic bands corresponding to species within the *L. plantarum* group could be detected after band extraction and DNA sequencing, apart from the fact that this species was perfectly detectable through the bacteria universal primers used here. Also after band extraction and sequencing, the presence of *Alkalibacterium* sp. was confirmed and *P. acidilactici* was detected. The last species had not been isolated in the previous study nor was it detected using universal primers.

PCR-DGGE analysis is known to only provide monitoring of main microbial populations (Murray et al., 1996; Muyzer et al., 1993). However, elucidation of DGGE profiles provides more information about the genetic structure of the dominant populations than the total microbial richness which is calculated through conventional culture-dependent techniques (Muyzer and Smalla, 1998). This is of special interest in complex microbiological systems such as natural table olive fermentations. In general terms, the results obtained through PCR-DGGE corroborated previous conclusions obtained by culture-dependent techniques. Thus, the prevalence of species belonging to the *L. plantarum* group was shown, what is in accordance with the ubiquitous isolation of *L. pentosus* in the previous study (Lucena-Padrós et al., 2014b). Other characteristic species were shown to be *patio* or stage specific (see Table 2). This is the case of *E. casseliflavus* and *Vibrio* sp. in *patio* 1, and *A. viridans/urinaeequi*, *E. olivae* and *P. parvulus*, more characteristic of *patio* 2. These results confirmed previous culture-dependent observations (Lucena-Padrós et al., 2014b). However, this study revealed the existence of new characteristic species, such as the ubiquitous *Alkalibacterium* sp. and *Marinilactibacillus* sp. Ntougias and Russell (2001) isolated *Alkalibacterium olivoapovliticus*

from wash waters after alkali treatment of table olives, but not from the olive fermentation itself, while *Marinilactibacillus* sp. has only been detected by culture-independent methods from table olive fermentations (Cocolin et al., 2013a). The fact that none of these species were detected in the cited previous study could be most probably due to the absence of specific culture media on that occasion. Although these halophilic/alkaliphilic bacteria were detected all throughout the fermentation, it is probable that actual viable cells were not present at the three stages considered, for it is known that they do not withstand low pH values (Yumoto et al., 2014). The use of culture-independent techniques such as RT-PCR-DGGE could contribute to obtain more realistic data about which species are playing a major role at each stage in these complex as well as long lasting food fermentations. Along with *Halolactibacillus* sp., detected for the first time in table olive fermentations in *patio* 2, these bacteria are characterized by their ability to carry out lactic acid fermentation under alkaline conditions, and this is why they are ascribed to the halophilic alkaliphilic lactic acid bacteria (HALAB) group (Yumoto et al., 2014). Current efforts in our laboratory are actually aimed to isolate this class of bacteria using specific culture media to assess their role in this table olive fermentation, especially at the initial stage.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2015.03.033.

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References

Abriouel, H., Benomar, N., Lucas, R., Gálvez, A., 2011. Culture-independent study of the diversity of microbial populations in brines during fermentation of naturally-fermented *Aloreña* green table olives. Int. J. Food Microbiol. 144, 487–496.

Amann, R., Kuhl, M., 1998. In situ methods for assessment of microorganisms and their activities. Curr. Opin. Microbiol. 1, 352–358.

Ampe, F., Ben Omar, N., Moizan, C., Wacher, C., Guyot, J.P., 1999. Polyphasic study of the spatial distribution of microorganisms in Mexican pozol, a fermented maize dough, demonstrates the need for cultivation-independent methods to investigate traditional fermentations. Appl. Environ. Microbiol. 65, 5464–5473.

Bernhard, A.E., Colbert, D., McManus, J., Field, K.G., 2005. Microbial community dynamics based on 16S rRNA gene profiles in a Pacific Northwest estuary and its tributaries. FEMS Microbiol. Ecol. 52, 115–128.

Cocolin, L., Manzano, M., Cantoni, C., Comi, G., 2001a. Denaturing gradient gel electrophoresis analysis of the 16S rRNA gene V1 region to monitor dynamic changes in the bacterial population during fermentation of Italian sausages. Appl. Environ. Microbiol. 67, 5113–5121.

Cocolin, L., Manzano, M., Aggio, D., Cantoni, C., Comi, G., 2001b. A novel polymerase chain reaction (PCR) denaturing gradient gel electrophoresis (DGGE) for the identification of *Micrococcaceae* strains involved in meat fermentations. Its application to naturally fermented Italian sausages. Meat Sci. 57, 59–64.

Cocolin, L., Alessandria, V., Dolci, P., Gorra, R., Rantsiou, K., 2013a. Culture independent methods to assess the diversity and dynamics of microbiota during food fermentation. Int. J. Food Microbiol. 167, 29–43.

Cocolin, L., Alessandria, V., Botta, C., Gorra, R., De Filippis, F., Ercolini, D., Rantsiou, K., 2013b. NaOH-debittering induces changes in bacterial ecology during table olives fermentation. PLoS One 8 (7), e69074. http://dx.doi.org/10.1371/journal.pone.0069074.

Giraffa, G., 2004. Studying the dynamics of microbial populations during food fermentation. FEMS Microbiol. Rev. 28, 251–260.

Giraffa, G., Neviani, E., 2001. DNA-based, culture-independent strategies for evaluating microbial communities in food-associated ecosystems. Int. J. Food Microbiol. 67, 19–34.

Heilig, H.G., Zoetendal, E.G., Vaughan, E., Marteau, E.P., Akkermans, A.D.L., de Vos, W.M., 2002. Molecular diversity of *Lactobacillus* spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. Appl. Environ. Microbiol. 68, 114–123.

Hugenholtz, P., Goebel, B.M., Pace, N.R., 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J. Bacteriol. 180, 4765–4774.

James, J.B., Sherman, T.D., Devereux, R., 2006. Analysis of bacterial communities in seagrass bed sediments by double-gradient denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA genes. Microb. Ecol. 52, 655–661.

Justé, A., Thomma, B.P.H.J., Lievens, B., 2008. Recent advances in molecular techniques to study microbial communities in food-associated matrices and processes. Food Microbiol. 25, 745–761.

Lane, D., 1991. 16S/23S rRNA sequencing. In: Stackebrandt, E., Goodfellow, M. (Eds.), Nucleic Acid Techniques in Bacterial Systematics. J. Wiley & Sons, Chichester, pp. 115–175.

Lucena-Padrós, H., Caballero-Guerrero, B., Maldonado-Barragán, A., Ruiz-Barba, J.L., 2014a. Microbial diversity and dynamics of Spanish-style green table-olive fermentations in large manufacturing companies through culture-dependent techniques. Food Microbiol. 42, 154–165.

Lucena-Padrós, H., González, J.M., Caballero-Guerrero, B., Ruiz-Barba, J.L., Maldonado-Barragán, A., 2014b. *Enterococcus olivae* sp. nov., isolated from Spanish-style green-olive fermentations. Int. J. Syst. Evol. Microbiol. 64, 2534–2539.

Lucena-Padrós, H., González, J.M., Caballero-Guerrero, B., Ruiz-Barba, J.L., Maldonado-Barragán, A., 2014c. *Propionibacterium olivae* sp. nov. and *Propionibacterium damnosum* sp. nov., isolated from spoiled packaged Spanish-style green olives. Int. J. Syst. Evol. Microbiol. 64, 2980–2985.

Lucena-Padrós, H., Caballero-Guerrero, B., Maldonado-Barragán, A., Ruiz-Barba, J.L., 2014d. Genetic diversity and dynamics of bacterial and yeast strains associated to Spanish-style green table-olive fermentations in large manufacturing companies. Int. J. Food Microbiol. 190, 72–78.

Martín, R., Heilig, H.G., Zoetendal, E.G., Jiménez, E., Fernández, L., Smidt, H., Rodríguez, J.M., 2007a. Cultivation-independent assessment of the bacterial diversity of breast milk among healthy women. Res. Microbiol. 158, 31–37.

Martín, R., Heilig, G.H.J., Zoetendal, E.G., Smidt, H., Rodríguez, J.M., 2007b. Diversity of the *Lactobacillus* group in breast milk and vagina of healthy women and potential role in the colonization of the infant gut. J. Appl. Microbiol. 103, 2638–2644.

Martín-Platero, A.M., Valdivia, E., Maqueda, M., Martínez-Bueno, M., 2007. Fast, convenient and economical method for isolating genomic DNA from lactic-acid bacteria using a modification of the protein "salting-out" procedure. Anal. Biochem. 366, 102–104.

Muccilli, S., Caggia, C., Randazzo, C.L., Restuccia, C., 2011. Yeast dynamics during the fermentation of brined green olives treated in the field with kaolin and Bordeaux mixture to control the olive fruit fly. Int. J. Food Microbiol. 148, 15–22.

Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G., Erlich, H., 1986. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. Cold Spring Harb. Symp. Quant. Biol. 51, 263–373.

Murray, A.E., Hollibaugh, J.T., Orrego, C., 1996. Phylogenetic compositions of bacterioplankton from two California estuaries compared by denaturing gradient gel electrophoresis of 16S rDNA fragments. Appl. Environ. Microbiol. 62, 2676–2680.

Muyzer, G., Smalla, K., 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Van Leeuwenhoek 73, 127–141.

Muyzer, G., de Waal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59, 695–700.

Ntougias, S., Russell, N.J., 2001. *Alkalibacterium olivoapovliticus* gen. nov., sp. nov., a new obligately alkaliphilic bacterium isolated from edible-olive wash-waters. Int. J. Syst. Evol. Microbiol. 51, 1161–1170.

Nubel, U., Engelen, B., Felske, A., Snaidr, J., Wieshuber, A., Amann, R.I., Ludwig, W., Backhaus, H., 1996. Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. J. Bacteriol. 178, 5636–5643.

Randazzo, C.L., Ribbera, A., Pitino, I., Romeo, F.V., Caggia, C., 2012. Diversity of bacterial population of table olives assessed by PCR-DGGE analysis. Food Microbiol. 32, 87–96.

Ringbauer, J.A., James, J.B., Genthner, F.J., 2006. Impact of large scale poultry farms on aquatic microbial communities: a molecular investigation. J. Water Health 4, 77–86.

Ruiz-Barba, J.L., Jiménez-Díaz, R., 2012. A novel *Lactobacillus pentosus*-paired starter culture for Spanish-style green olive fermentation. Food Microbiol. 30, 253–259.

Ruiz-Barba, J.L., Maldonado, A., Jiménez-Díaz, R., 2005. Small-scale total DNA extraction from bacteria and yeast for PCR applications. Anal. Biochem. 347, 333–335.

Sanguinetti, C.J., Dias-Neto, E., Simpson, A.J., 1994. Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. BioTechniques 17, 914–921.

Sigler, W.V., Miniaci, C., Zeyer, J., 2004. Electrophoresis time impacts the denaturing gradient gel electrophoresis-based assessment of bacterial community structure. J. Microbiol. Methods 57, 17–22.

Sokal, R.R., Rohlf, F.J., 1987. Introduction to Biostatistics. W.H. Freeman and Company, New York, pp. 333–337.

van Hannen, E.J., Zwart, G., van Agterveld, M.P., Gons, H.J., Ebert, J., Laanbroek, H.J., 1999. Changes in bacterial and eukaryotic community structure after mass lysis of filamentous cyanobacteria associated with viruses. Appl. Environ. Microbiol. 65, 795–801.

Yumoto, I., Hirota, K., Nakajima, K., 2014. The genus *Alkalibacterium*. In: Holzapfel, W.H., Wood, B.J.B. (Eds.), Lactic Acid Bacteria: Biodiversity and Taxonomy. John Wiley & Sons, Oxford, UK, pp. 147–156.

Table S1. Assignment of banding patterns to location (*patio*) groups of the bacterial community in Spanish-style olive fermentations by jackknife analysis (Dice's coefficient).

	Banding patterns (%) assigned to groups	
Location	*Patio* 1 (n=60)	*Patio* 2 (n=66)
Patio 1	**41.7**	0.0
Patio 2	58.3	**100**

ERCCs of patterns to host class are in boldface. The mean ERCC was 72.22 %.

Table S2. Assignment of banding patterns to fermentation stage groups of the bacterial community in Spanish-style olive fermentations by jackknife analysis (Dice's coefficient).

	Banding patterns (%) assigned to groups		
Fermentation stage	Initial (n=40)	Middle(n=43)	Final (n=43)
Initial	**52.5**	7.0	0.0
Middle	45.0	**60.5**	27.9
Final	2.5	32.6	**72.1**

ERCCs of patterns to host class are in boldface. The mean ERCC was 61.90 %.

Table S3. Assignment of banding patterns to both location (*patio*) and fermentation stage groups of the bacterial community in Spanish-style olive fermentations by jackknife analysis (Dice's coefficient).

Patio-Stage	Banding patterns (%) assigned to groups					
	1-Initial (n = 20)	1-Middle (n = 20)	1-Final (n = 20)	2-Initial (n = 20)	2-Middle (n = 23)	2-Final (n = 23)
1-Initial	**90.0**	15.0	0.0	0.0	0.0	0.0
1-Middle	0.0	**35.0**	15.0	0.0	0.0	0.0
1-Final	0.0	15.0	**25.0**	0.0	0.0	0.0
2-Initial	0.0	0.0	0.0	**55.0**	17.4	0.0
2-Middle	10.0*	20.0	5.0*	45.0	**47.8**	17.4
2-Final	0.0	15.0*	55.0	0.0	34.8	**82.6**

ERCCs of patterns to host class are in boldface. The mean ERCC was 56.35%. *Incorrect banding assigments considering both factors, i.e. location (*patio*) and fermentation stage (4.76 % of the samples).

Table S4. Assignment of banding patterns to area groups of the *Lactobacillus*-group community in Spanish-style olive fermentations in *patio* 2 by jackknife analysis (Dice's coefficient).

	Banding patterns (%) assigned to groups		
Area	2a (n = 24)	2b (n = 36)	2c (n = 6)
2a	**8.3**	5.6	0
2b	8.3	**38.9**	0
2c	83.3	55.6	**100**

ERCCs of patterns to host class are in boldface. The mean ERCC was 33.32%, while the random ERCC was 54.55%. Differences are statistically significant ($p < 0.05$)

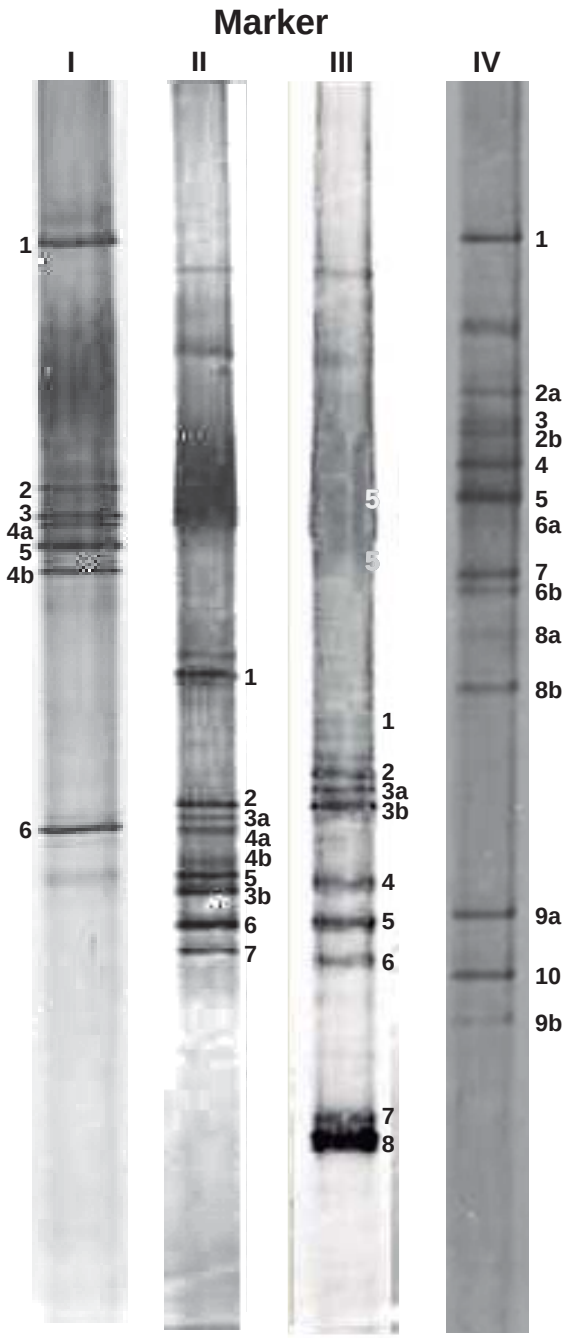
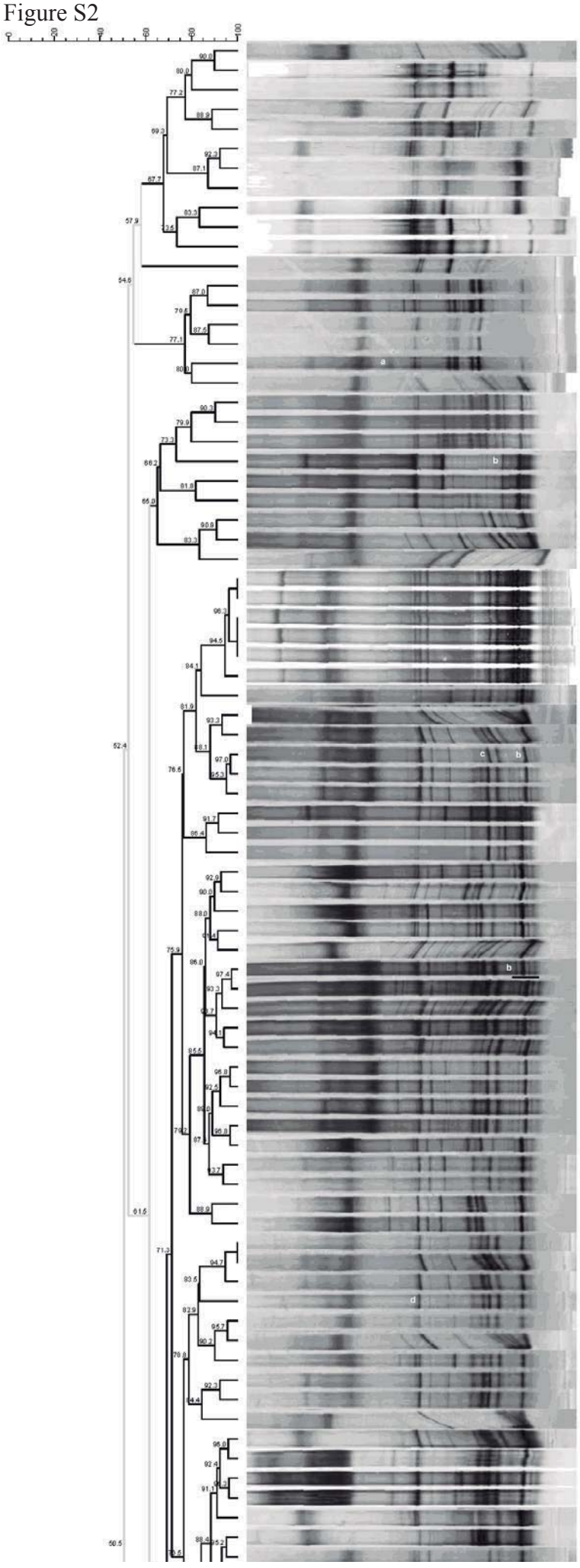


Figure S1. PCR-DGGE profiles of the 16S rDNA amplicons using universal bacteria primers (Markers I, II and III) as well as *Lactobacillus* group-specific primers (Marker IV) of reference bacterial species which were used to construct the respective DGGE markers. Marker I: 1, *Staphylococcus sp.* (epidermidis group); 2, *C. xylanovorans*; 3, *C. jejuense;* 4, *C. schirmacherense*/*argentinense;* 5, *C. sporogenes;* 6, *C. tyrobutyricum*. Marker II: 1, *L. pentosus;* 2, *L. parafarraginis;* 3, *L. collinoides/paracollinoides;* 4, *P. parvulus*; 5, *L. paracasei;* 6, *P. ethanolidurans;* 7, *L. coryniformis*. Marker III:1, *A. viridans*; 2, *E. casseliflavus*; 3, *W. paramesenteroides/hellenica*; 4, *E. faecium*; 5, *E. faecalis*; 6, *E. olivae*; 7, *P. olivae*; 8, *P. acnes*. Marker IV: 1, *L. lactis spp. lactis;* 2, *L. mesenteroides;* 3, *P. parvulus;* 4, *P. acidilactici;* 5, *L. rapi;* 6, *L. collinoides/paracollinoides;* 7, *L. parafarraginis*; 8, *L. rhamnosus;* 9, *L. paracasei.* Numbers along the lanes indicate those bands taken as representative of a specific bacterial species. When more than one single band has been considered for a given species, it is indicated by letters.

Figure S2



Fermenter	Patio	Area	Stage
2	2	2a	Initial
11	1	1b	Final
11	1	1b	Middle
19	1	1a	Middle
13	1	1b	Middle
14	1	1b	Final
15	1	1b	Final
15	1	1b	Middle
14	1	1b	Middle
13	1	1b	Final
12	1	1b	Final
2	1	1a	Middle
5	1	1a	Middle
5	1	1a	Final
2	1	1a	Final
8	1	1a	Final
8	1	1a	Middle
6	1	1a	Final
17	1	1a	Middle
17	1	1a	Final
17	1	1a	Initial
7	1	1a	Initial
7	1	1a	Middle
7	1	1a	Final
1	1	1a	Middle
1	1	1a	Final
19	1	1a	Initial
21	2	2c	Middle
23	2	2c	Final
22	2	2c	Middle
23	2	2c	Middle
22	2	2c	Final
21	2	2c	Final
3	1	1a	Middle
10	2	2b	Initial
9	2	2b	Initial
6	2	2a	Initial
8	2	2a	Initial
7	2	2a	Initial
10	1	1a	Initial
10	1	1a	Middle
10	1	1a	Final
4	2	2a	Middle
4	2	2a	Final
3	2	2a	Middle
3	2	2a	Final
5	2	2a	Final
10	2	2b	Final
6	2	2a	Final
8	2	2a	Final
9	2	2b	Final
7	2	2a	Final
6	2	2a	Middle
7	2	2a	Middle
10	2	2b	Middle
8	2	2a	Middle
5	2	2a	Middle
12	2	2b	Initial
12	2	2b	Middle
4	2	2a	Initial
5	2	2a	Initial
17	2	2b	Middle
18	2	2b	Middle
18	2	2b	Initial
16	2	2b	Middle
17	2	2b	Final
18	2	2b	Final
16	2	2b	Final
16	2	2b	Initial
17	2	2b	Initial
1	2	2a	Initial
14	2	2b	Middle
13	2	2b	Final
19	2	2b	Final
20	2	2b	Final
14	2	2b	Final
15	2	2b	Middle
12	2	2b	Final

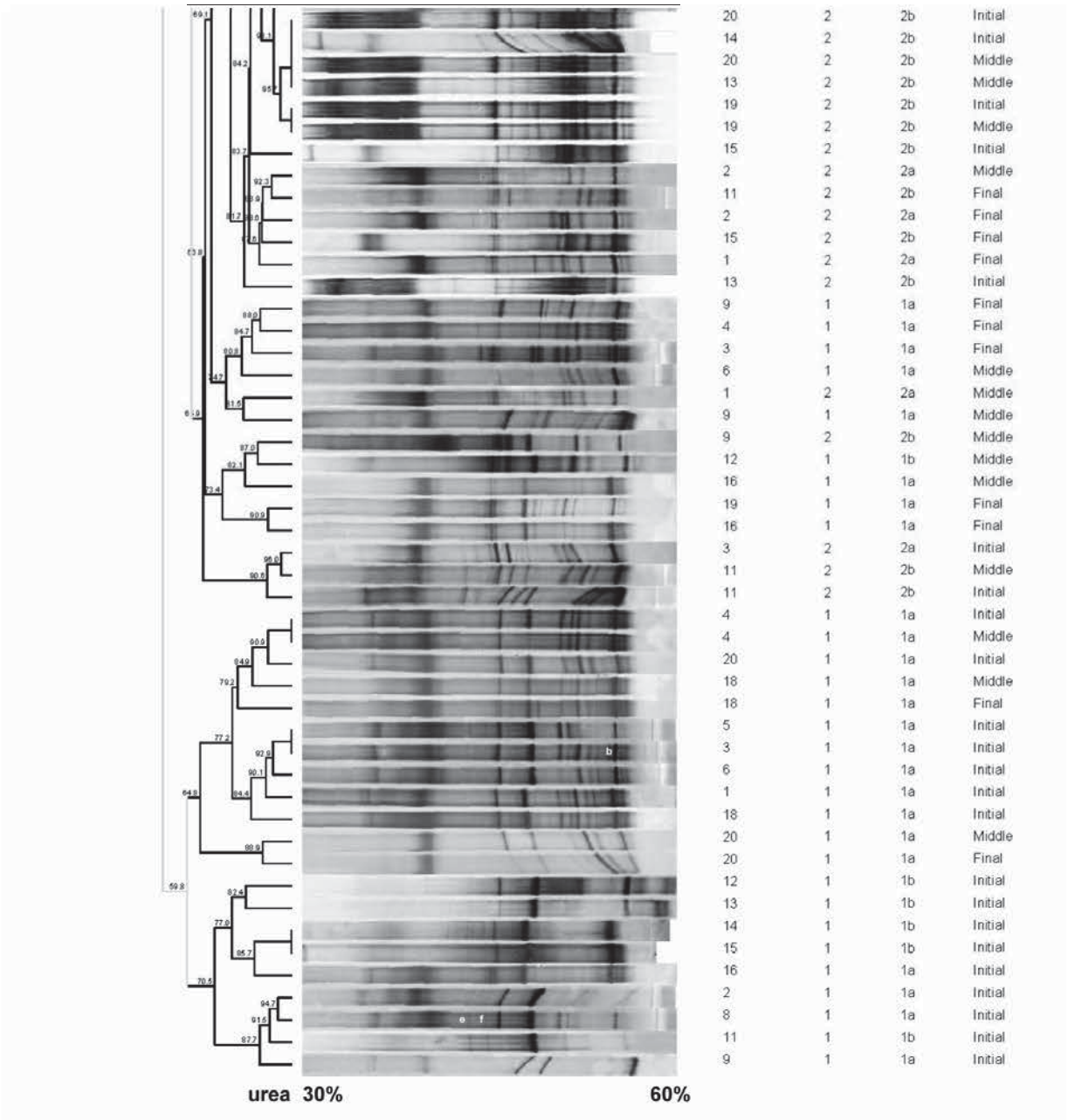


Figure S2. Phylogenetic dendrogram obtained from PCR-DGGE profiles using bacteria universal primers during Spanish-style green olive fermentations in two fermentation yards (patios). UPGMA clustering algorithm and Dice's coefficient of similarity were used. The fermenter from which the sample was taken, as well as the patio, the area it was located within each patio and the fermentation stage are indicated. Scale line at the top indicates the percentage of similarity. The classes of bands identified by gel excision and subsequent DNA sequencing are indicated at the left side of representative bands as follows: a, *Lactobacillus collinoides/paracollinoides*; b, *Alkalibacterium* sp. (the black bar indicates a group of four characteristic bands for species within this genus); c, *Marinilactibacillus* sp.; d, *Halolactibacillus* sp.; e, *Vibrio* sp.; f, *Vibrio furnisii/fluvialis*. DGGE analysis was performed in the range 30-60% urea, as indicated.

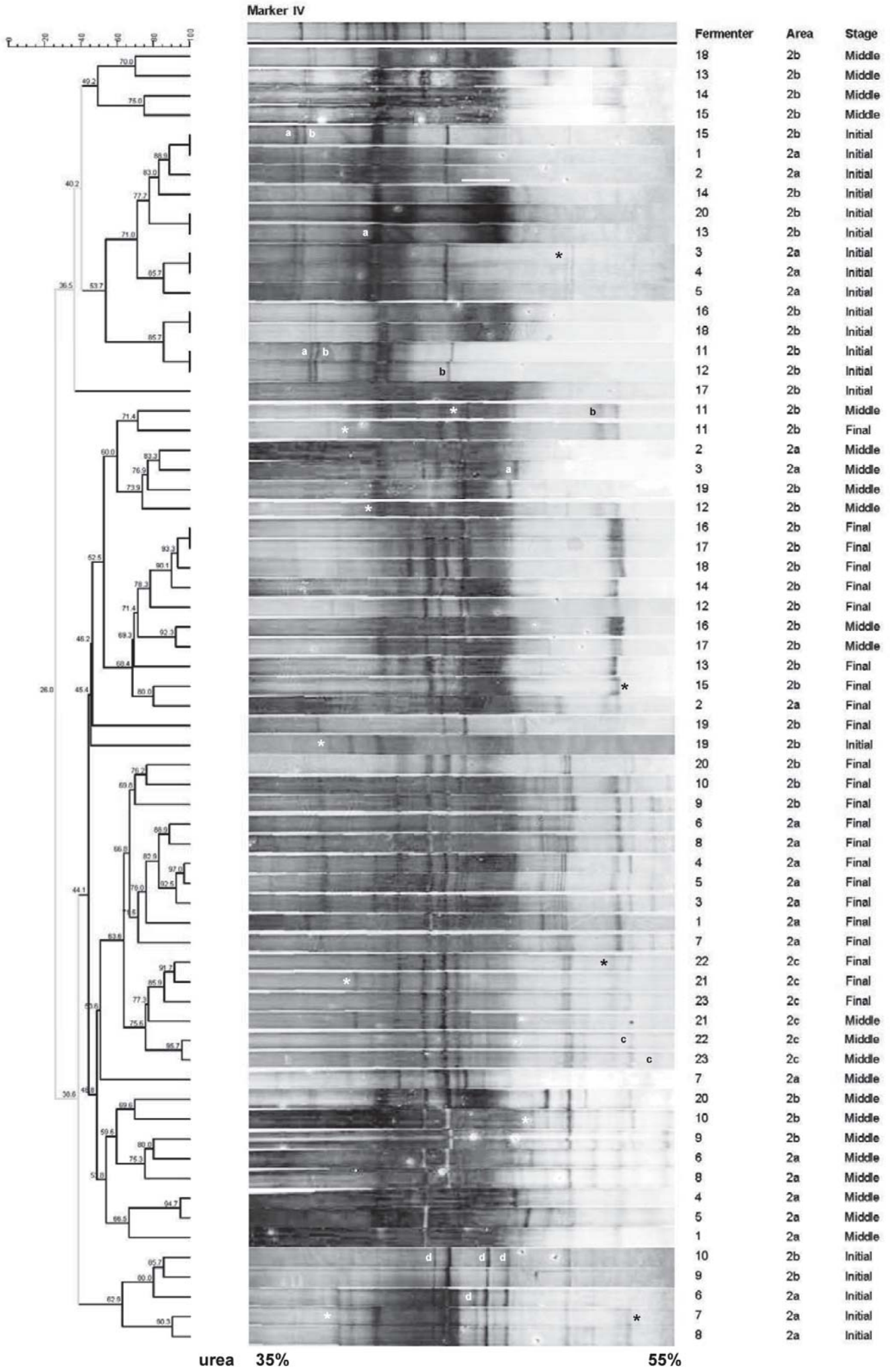

Marker IV
Fermenter Area Stage
18 2b Middle
13 2b Middle
14 2b Middle
15 2b Middle
15 2b Initial
1 2a Initial
2 2a Initial
14 2b Initial
20 2b Initial
13 2b Initial
3 2a Initial
4 2a Initial
5 2a Initial
16 2b Initial
18 2b Initial
11 2b Initial
12 2b Initial
17 2b Initial
11 2b Middle
11 2b Final
2 2a Middle
3 2a Middle
19 2b Middle
12 2b Middle
16 2b Final
17 2b Final
18 2b Final
14 2b Final
12 2b Final
16 2b Middle
17 2b Middle
13 2b Final
15 2b Final
2 2a Final
19 2b Final
19 2b Initial
20 2b Final
10 2b Final
9 2b Final
6 2a Final
8 2a Final
4 2a Final
5 2a Final
3 2a Final
1 2a Final
7 2a Final
22 2c Final
21 2c Final
23 2c Final
21 2c Middle
22 2c Middle
23 2c Middle
7 2a Middle
20 2b Middle
10 2b Middle
9 2b Middle
6 2a Middle
8 2a Middle
4 2a Middle
5 2a Middle
1 2a Middle
10 2b Initial
9 2b Initial
6 2a Initial
7 2a Initial
8 2a Initial
urea 35% 55%

Figure S3

Figure S3. Phylogenetic dendrogram obtained from PCR-DGGE profiles using *Lactobacillus*-group specific primers during Spanish-style green olive fermentations in fermentation yard (*patio*) #2. UPGMA clustering algorithm and Dice's coefficient of similarity were used. The fermenter from which the sample was taken, as well as the area it was located within *patio* 2 and the fermentation stage are indicated. Electrophoretic bands which were excised from the gel and identified through DNA sequencing are indicated as follows: a, *Lactobacillus plantarum* group; b, *Aerococcus viridans*; c, *Lactobacillus collinoides/paracollinoides*; d, *Alkalibacterium* sp. The white bar indicates the usual location of a "fuzzy band" which is characteristic of some strains belonging to the *L. plantarum* group.*, Electrophoretic bands which did not match any in the markers and were extracted from the gels, purified and sequenced with no conclusive results. Scale line at the top indicates the percentage of similarity. DGGE analysis was performed in the range 35-55% urea, as indicated.

4.6 *Vibrio olivae* sp. nov., isolated from Spanish-style green-olive fermentations.

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Vibrio olivae sp. nov., isolated from Spanish-style green-olive fermentations

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Three isolates originating from Spanish-style green-olive fermentations in a manufacturing company in the province of Seville, Spain, were taxonomically characterized by a polyphasic approach. This included a phylogenetic analysis based on 16S rRNA gene sequences and multi-locus sequence analysis (MLSA) based on *pyrH*, *recA*, *rpoA*, *gyrB* and *mreB* genes. The isolates shared 98.0 % 16S rRNA gene sequence similarity with *Vibrio xiamenensis* G21^T. Phylogenetic analysis based on 16S rRNA gene sequences using the neighbour-joining and maximum-likelihood methods showed that the isolates fell within the genus *Vibrio* and formed an independent branch close to *V. xiamenensis* G21^T. The maximum-parsimony method grouped the isolates to *V. xiamenensis* G21^T but forming two clearly separated branches. Phylogenetic trees based on individual *pyrH*, *recA*, *rpoA*, *gyrB* and *mreB* gene sequences revealed that strain IGJ1.11^T formed a clade alone or with *V. xiamenensis* G21^T. Sequence similarities of the *pyrH*, *recA*, *rpoA*, *gyrB* and *mreB* genes between strain IGJ1.11^T and *V. xiamenensis* G21^T were 86.7, 85.7, 97.3, 87.6 and 84.8 %, respectively. MLSA of concatenated sequences showed that strain IGJ1.11^T and *V. xiamenensis* G21^T are two clearly separated species that form a clade, which we named Clade *Xiamenensis*, that presented 89.7 % concatenated gene sequence similarity, i.e. less than 92 %. The major cellular fatty acids (>5 %) of strain IGJ1.11^T were summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$), $C_{16:0}$ and summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$). Enzymic activity profiles, sugar fermentation patterns and DNA G+C content (52.9 mol%) differentiated the novel strains from the closest related members of the genus *Vibrio*. The name *Vibrio olivae* sp. nov. is proposed for the novel species. The type strain is IGJ1.11^T (=CECT 8064^T=DSM 25438^T).

At the time of writing, the genus *Vibrio* consisted of 111 species according to the List of Prokaryotic Names with Standing in Nomenclature (LPSN; http://www.bacterio.net). Recently, species of the genus *Vibrio* have been grouped in 19 clades based on multi-locus sequence analysis (MLSA) using eight housekeeping genes (Sawabe *et al.*, 2013). Vibrios are Gram-negative, usually motile rods, halophilic, mesophilic and chemo-organotrophic, and have a facultatively fermentative metabolism (Baumann *et al.*, 1984). Species from this genus have been commonly isolated from aquatic environments, usually from marine environments, both as free-living bacteria and as symbionts or parasites of fish, molluscs and crustaceans (Thompson *et al.*, 2004).

Spanish-style green-olive fermentations are characterized by an ecological succession of diverse microbial species. Their preparation is carried out by an initial alkali treatment [1.8–3.5 % (w/v) NaOH] of the green fruits, in order to remove bitterness and allow the subsequent growth of lactic acid bacteria (LAB) through the neutralization and

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Abbreviations: LAB, lactic acid bacteria; ML, maximum-likelihood; MLSA, multi-locus sequence analysis; MP, maximum-parsimony; NJ, neighbour-joining; RAPD, random amplified polymorphic DNA.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *pyrH*, *recA*, *rpoA*, *gyrB* and *mreB* gene sequences of strain IGJ1.11^T are JQ283456, JQ283457, JQ283458, JQ283459, KP162090 and KP162091, respectively.

Three supplementary tables and nine supplementary figures are available with the online Supplementary Material.

washing of inhibitory phenolic compounds (Rejano *et al.*, 2010). Once the alkali is removed, fruits are washed once or twice with water and finally covered with brine [10–12 % (w/v) NaCl]. In this brine, a spontaneous fermentation takes place in which at least three different stages have been identified (Fernandez *et al.*, 1995). During the first stage, usually lasting 3–10 days, fermentation is conducted by the indigenous alkali-tolerant microbiota that contaminates the fruits as well as the environment (de Castro *et al.*, 2002). This microbiota is responsible for lowering the initial high pH (10–11) to values close to 6–7, more appropriate for the growth of LAB, which are also present as contaminants (Sanchez *et al.*, 2001). Later, the usual alkali-tolerant microbiota disappear as a consequence of the lowering of the pH due to the growth of LAB, mainly strains of *Lactobacillus pentosus*, which is characteristic of the second fermentation stage (de Castro *et al.*, 2002; Rejano *et al.*, 2010; Ruiz-Barba & Jiménez-Diaz, 2012). In the final stage of the fermentation, sugars are exhausted and LAB population declines steadily, thus starting the storage period.

Recently, we have carried out an exhaustive analysis of the microbiota associated to Spanish-style green olive fermentations, attending to its dynamics along the time (Lucena-Padrós *et al.*, 2014). During this study, we found nine isolates at the initial stage of the fermentation whose partial 16S rRNA gene sequences showed similarity (≤97 %) to the species *Vibrio furnissii* and *Vibrio fluvialis*, suggesting that they could constitute a novel species of the genus *Vibrio*. As stated above, this first stage of the fermentation process is characterized by high pH and salt values, which is an ideal environment for the grown of alcaliphilic and halophilic bacteria such as species of the genus *Vibrio*.

The nine isolates were recovered from brine samples from six different 10-tonne fermenters at the first two weeks of the fermentation process as described previously (Lucena-Padrós *et al.*, 2014). Briefly, samples were serially diluted and spread onto plates of brain heart infusion (BHI; Biokar Diagnostics) supplemented with 0.05 % (w/v) L-cysteine (AppliChem). Plates were incubated anaerobically at 30 °C for 48 h in a DG250 Anaerobic Workstation (Don Whitley Scientific), with a gas mixture consisting of 10 % H_2/10 % CO_2/80 % N_2.

The nine isolates, were genotyped by random amplified polymorphic DNA (RAPD) analysis using the primer ISS1rev (5′-GGATCCAAGACAACGTTTCAAA-3′) (Veyrat *et al.*, 1999), following the protocol of Maldonado-Barragan *et al.* (2013). Three representative isolates (IGJ1.11^T, IGJ8.11 and IGJ10.11) showing a different RAPD profile were selected for further characterization (Fig. S1, available in the online Supplementary Material).

DNA from pure cultures was isolated according to the protocol of Lazo *et al.* (1987). A fragment of the 16S rRNA gene was amplified with the primer pair 7 for (5′-AGAGTTTGATYMTGGCTCAG-3′) and 1510r (5′-TACGGYTACCTTGTTACGACTT-3′) (Lane, 1991). The *rpoA*, *recA*, *gyrB*, *pyrH* and *mre*B genes were amplified for MLSA according to Thompson *et al.* (2005) and Sawabe *et al.* (2007). The identification of the isolates and their phylogenetic neighbours was carried out by the BLASTN program on the basis of 16S rRNA gene sequence data obtained (Altschul *et al.*, 1997) against the database containing type strains with updated validly published prokaryotic names, by using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim *et al.*, 2012). The calculation of pairwise sequence similarity was done using the global alignment algorithm (Myers & Miller, 1988), which was implemented at the EzTaxon-e server. The *pyrH*, *recA*, *rpoA*, *gyrB* and *mreB* nucleotide sequences similarities were determined using the MEGALIGN software in the DNASTAR package. The DNA sequences belonging to type strains of species of the genus *Vibrio* were obtained from the GenBank database.

To test the evolutionary relationships of the genus *Vibrio*, phylogenetic analysis based on individual and concatenated sequences was performed. For this, the sequences were aligned by using the CLUSTAL W method (Thompson *et al.*, 1994) with the MEGA 5 (version 5.2) software (Tamura *et al.*, 2011). To reconstruct the phylogenetic trees based on individual and concatenated *pyrH*, *recA*, *rpoA*, *gyrB* and *mreB* genes, we used regions 172–577, 440–937, 86–875, 441–1035 and 386–923 [*V. cholerae* O1 Eltor N16961 (GenBank accession no. AE003852) numbering], respectively. These regions were in the range of those used by Sawabe *et al.* (2007, 2013). The phylogenetic trees were reconstructed based on the neighbour-joining (NJ) (Saitou & Nei, 1987), maximum-parsimony (MP) (Fitch, 1971) and maximum-likelihood (ML) (Felsenstein, 1981) methods. For NJ and ML analysis, distance matrices were calculated using Kimura's two-parameter correction. ML analysis was carried out using a heuristic search option (Nearest-Neighbour-Interchange; NNI). MP analysis was performed using the Subtree-Pruning-Regrafting (SPR) search method. Bootstrapping analysis (1000 replicates) was done to study the stability of the groupings. The GenBank accession numbers for genes used in the MLSA are given in Table S1.

The degree of DNA–DNA relatedness between strain IGJ1.11^T and its closest phylogenetic neighbour, *V. xiamenensis* DSM 22851^T (=G21^T) was determined by the fluorimetric method as described by Gonzalez & Saiz-Jimenez (2005). This method measures the divergence between the thermal denaturation midpoint of homoduplex DNA and heteroduplex DNA (ΔT_m) using a realtime PCR thermocycler that obtains fluorescence determinations. The G+C content of genomic DNA of strain IGJ1.11^T was also determined by the fluorimetric method described by Gonzalez & Saiz-Jimenez (2002). The results of both DNA–DNA relatedness and DNA G+C content were expressed as mean percentage values ±SD, based on eight and three independent experiments, respectively.

Substrate utilization, the sugar fermentation/oxidation profile, acid production and other biochemical characteristics were examined at the Spanish type Culture Collection

(CECT; Universitat de València, Paterna, Valencia, Spain) using the API 20 E fermentation kit (bioMérieux) after incubation for 24 h at 30 °C, according to the manufacturer's instructions. Cell morphology, size and motility were examined at the CECT using a phase-contrast Leica DMRB microscope. Cells of a 14 h-old culture grown at 30 °C on marine agar (MA) were examined. Micrographs were taken using a Leica EC3 digital camera and processed using the software Leica LAS EZ (Leica Microsystems). Cell size was estimated from a digital image calculating mean (n = 10) length and width. The temperature range for growth was determined by culturing the isolate in MA at 4–45 °C. Growth at pH 4.0–10.0 was tested at 30 °C in BHI-cys with the pH adjusted with HCl (for pH 4.0–7.0) or NaOH (for pH 7.0–10.0). Sensitivity of growth to NaCl concentration was determined with modified BHI-cys, in which the concentration of NaCl ranged from 1 to 10 % (w/v). Growth under anaerobic conditions was determined by incubation on BHI-cys as described above. Growth on thiosulfate-citrate-bile-sucrose (TCBS; Difco) plates was determined at 30 °C. Catalase activity was determined by bubble production in a 10 % (v/v) H_2O_2 solution.

Analysis of whole-cell fatty acids was carried out at CECT following the protocol recommended by the MIDI Microbial Identification System (Sasser, 1990) using a culture grown on MA for 14 h at 30 °C. Analysis of fatty acid methyl esters was carried out with an Agilent Technologies 6850 gas chromatograph with the Sherlock 6.1 database using the Sherlock Microbial Identification System (MIDI).

The 16S rRNA gene sequences of isolates IGJ1.11^T, IGJ8.11 and IGJ10.11 were 100 % identical. The BLASTN analysis of these 16S rRNA gene sequences (1437 bp) showed that isolates IGJ1.11^T, IGJ8.11 and IGJ10.11 belong to the genus *Vibrio*. The type strains of the most closely related species of the genus *Vibrio* were *V. xiamenensis* G21^T, *V. furnissii* CIP 102972^T, *V. fluvialis* NCTC 11327^T, *V. variabilis* R-40492^T and *Vibrio hepatarius* LMG 20362^T, which showed 98.0, 97.6, 97.4, 97.2 and 97.1 % 16S rRNA gene sequence similarity, respectively (Table S2). Recently, Kim *et al.* (2014) have proposed 98.65 % 16S rRNA gene sequence similarity as the threshold for differentiating two species, being in the threshold range (98.2–99.0 %) previously suggested by other studies (Meier-Kolthoff *et al.*, 2013; Stackebrandt & Ebers, 2006). The 16S rRNA gene sequence similarity between isolates IGJ1.11^T, IGJ8.11 and IGJ10.11 and *V. xiamenensis* G21^T (98.0 %) is below 98.65 %, thus indicating the isolates represent a distinct species.

The phylogenetic analysis inferred from the 16S rRNA sequences, using the NJ and ML methods, showed that the isolate IGJ1.11^T forms an independent branch close to *V. xiamenensis* G21^T (Fig. 1 and Fig. S2). The MP method grouped isolate IGJ1.11^T to *V. xiamenensis* G21^T;

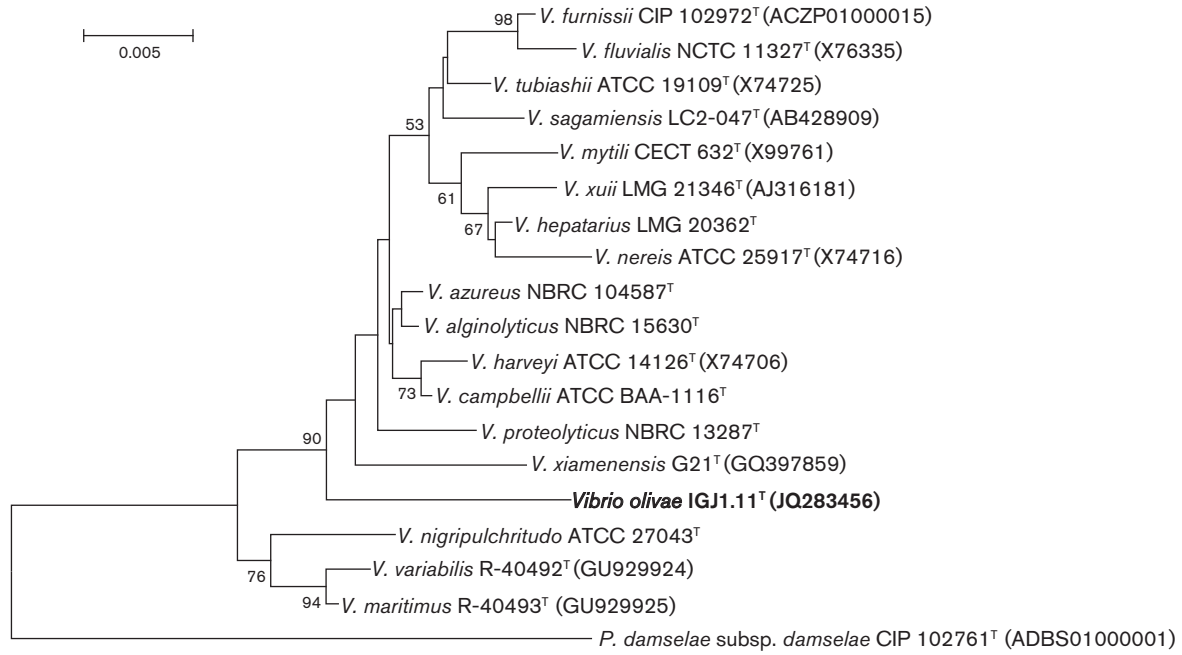

Fig. 1. Phylogenetic tree based on almost-complete 16S rRNA gene sequences showing the relationship of strain IGJ1.11^T to some members of the genus *Vibrio*. The tree was reconstructed using the neighbour-joining method. Numbers on the tree indicate bootstrap values calculated for 1000 subsets for branch-points >50 %. *Photobacterium damselae* subsp. *damselae* CIP 102761^T was used as an outgroup. GenBank sequence accession numbers are given in parentheses. Bar, 0.005 nt changes per nucleotide position.

however, bootstrap resampling values (64 %) support that isolate IGJ1.11^T and *V. xiamenensis* G21^T form two clearly separated branches (Fig. S3).

Nowadays, MLSA is considered a reliable technique to reconstruct the phylogeny of the genus *Vibrio* (Thompson *et al.*, 2005; Sawabe *et al.*, 2007, 2013). The *pyrH, recA, rpoA, gyrB and mreB* gene sequences of the isolates IGJ1.11^T, IGJ8.11 and IGJ10.11 were 100 % identical. Phylogenetic trees based on *pyrH, recA, rpoA, gyrB and mreB* gene sequences revealed that strain IGJ1.11^T formed a clade alone or with *V. xiamenensis* (Figs S4–S8). Sequence similarities of the *pyrH, recA, rpoA, gyrB and mreB* genes between strain IGJ1.11^T and *V. xiamenensis* G21^T were 86.7, 85.7, 97.3, 87.6, and 84.8 %, respectively (Table S2). These values are below the threshold values of 98 %, 94 % and 94 % for *rpoA*, *recA* and *pyrH*, respectively, recommended for delineating species in the genus *Vibrio* (Thompson *et al.*, 2005; Sawabe *et al.*, 2007).

The phylogenetic tree based on concatenated sequences *pyrH, recA, rpoA, gyrB and mreB*, showed that isolate IGJ1.11^T forms a clade with *V. xiamenensis* G21^T (Fig. 2). Bootstrap resampling values (100 %) indicated that the novel isolates and *V. xiamenensis* are two clearly separated species. Interestingly, all species of the genus *Vibrio* used in this study were grouped into their corresponding clade according to the recent update of the *Vibrio* clades carried out by Sawabe *et al.* (2013) (Fig. 2). In the light of the phylogenetic analysis based on MLSA of five concatenated sequences (*pyrH, recA, rpoA, gyrB and mreB*), we propose that strainIGJ1.11^T represents a novel species which, together with *V. xiamenensis* G21^T, could form a new clade, i.e. the clade *Xiamenensis* (Fig. 2).

Thompson *et al.* (2005) suggested that species of the genus *Vibrio* are defined as a group of strains that share more than 95 % similarity among MLSA datasets. Recently, Sawabe *et al.* (2013) showed that <98 % of 8-gene-concatenated nucleotide sequence similarity may allow us to define a species boundary. Thus, strains below this threshold value would qualify as members of separate species. Strain IGJ1.11^T showed an 89.7 % gene-concatenated nucleotide sequence similarity to

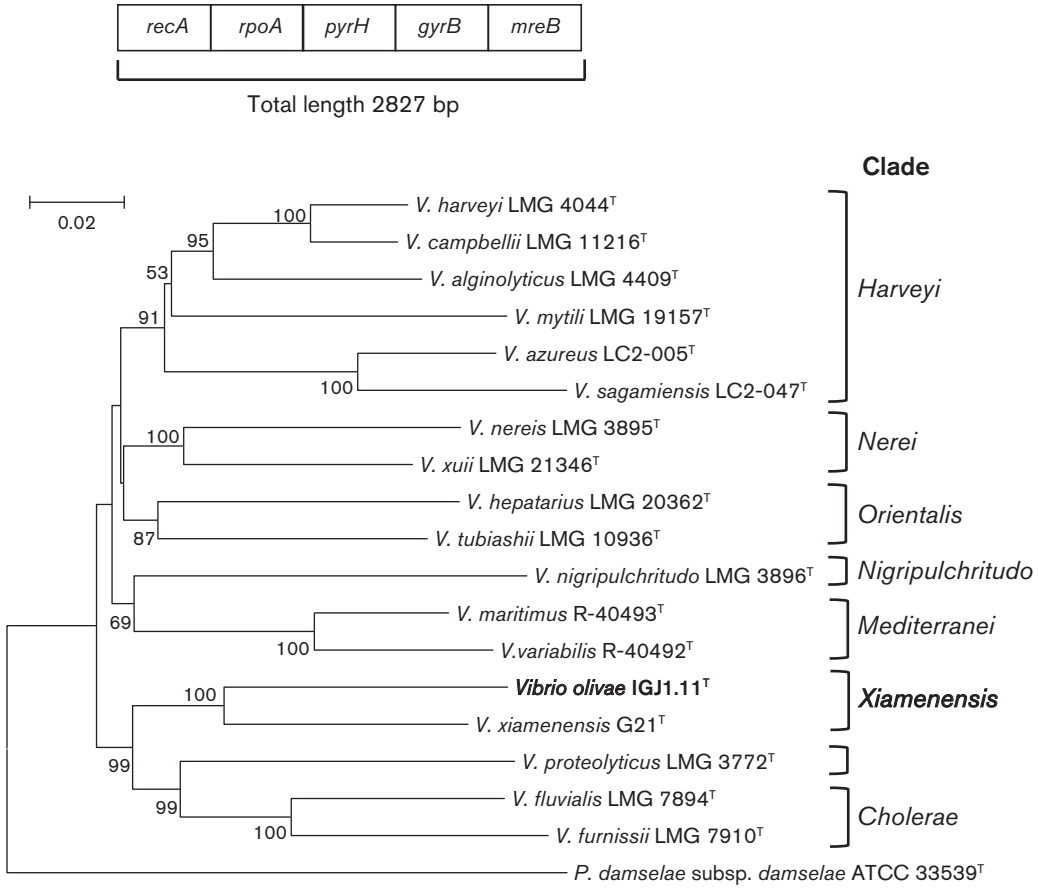

Fig. 2. Phylogenetic tree based on concatenated sequences of the *recA* (498 bp), *rpoA* (790 bp), *pyrH* (406 bp), *gyrB* (595 bp) and *mreB* (538 bp) genes, using the neighbour-joining method. Bootstrap values were expressed based on 1000 replications; only values ≥50 % are shown. Clades to which the different species belong are indicated. *Photobacterium damselae* subsp. *damselae* ATCC 33539^T was used as an outgroup. Bar, 2 % estimated sequence divergence.

its nearest neighbour, *V. xiamenensis* G21^T, thus indicating it represents a novel species of the genus *Vibrio* (Table S2).

The difference in melting temperature between genomic DNA from strain IGJ1.11^T and that from *V. xiamenensis* DSM 22851^T (=G21^T) was 6.4 °C (SD=1.0; n = 8). This value was above the 5 °C ΔT_m recommended as cut-off point for the delineation of species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). DNA–DNA relatedness results confirmed that strain IGJ1.11^T represents a novel species clearly differentiated from *V. xiamenensis*. The G+C content of genomic DNA of strain IGJ1.11^T was 52.9 mol% (SD=0.95, n = 3), which was within the range of the genus *Vibrio*.

The three analysed isolates (IGJ1.11^T, IGJ8.11 and IGJ10.11) were shown to possess the same biochemical profile described in Table 1 for the type strain, except for citrate utilization, where strain IGJ1.11^T was positive but strains IGJ8.11 and IGJ 10.11 were negative. Table 1 shows phenotypical tests that are useful for the differentiation of strain IGJ1.11^T from the closest phylogenetic relatives, i.e. *V. xiamenensis*, *V. fluvialis*, *V. furnissii* and *V. proteolyticus*. Strain IGJ1.11^T (and strains IGJ8.11 and IGJ10.11) could be easily differentiated from its neighbours by the ability to produce acid from melibiose.

Table 1. Biochemical and metabolic characteristics that differentiate strain IGJ1.11^T from related species of the genus *Vibrio*

Strains: 1, IGJ1.11^T; 2, *V. xiamenensis* G21^T; 3, *V. furnissii* CAIM 518^T; 4, *V. fluvialis* CAIM 593^T; 5, *V. proteolyticus* NBRC 13287^T. Data for reference type strains were taken from Brenner *et al.* (1983), Gao *et al.* (2012), Farmer & Hickman-Brenner (2006), Farmer *et al.* (2005), Kalina *et al.* (1984) and Noguerola & Blanch (2008). Biochemical characteristics of strain IGJ1.11^T were determined using the API 20E identification system (bioMérieux). Growth of strain IGJ1.11^T on TCBS plates was determined at 30 °C, while growth in 10% NaCl was determined in BHI-cys at 30 °C. The G+C content of genomic DNA of strain IGJ1.11^T was determined by the fluorimetric method of Gonzalez & Saiz-Jimenez (2002). All strains were negative for H_2S production. +, Positive; –, negative; V, variable; ND, no data available. ^{y}, yellow colonies.

Characteristic	1	2	3	4	5
β-Galactosidase	+	+	−	+	−
Arginine dihydrolase	−	−	+	+	+
Lysine decarboxylase	−	−	−	−	+
Citrate utilization	+	−	+	+	+
Urease	−	−	−	−	V
Indole production	−	−	−	−	+
Voges–Proskauer	−	−	−	−	+
Gelatinase	+	−	+	+	+
Catalase	−	+	ND	ND	−
Acid production from:					
D-Sorbitol	−	−	−	−	+
L-Rhamnose	+	+	+	−	−
D-Sucrose	+	+	+	+	−
D-Melibiose	+	−	−	−	−
Amygdalin	+	+	ND	ND	−
L-Arabinose	+	+	+	+	−
Growth in/on:					
10% (w/v) NaCl	+	+	−	−	+
TCBS medium	+^{y}	−	+^{y}	+^{y}	ND
DNA G+C content (mol%)	52.9	46.0	50.4	49.3–50.3	50.5

Growth at different temperatures, pH and NaCl concentrations and other phenotypical features are given in the species description. The major cellular fatty acids (>5%) of strain IGJ1.11^T were summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$), $C_{16:0}$ and summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$). Although similar to other species of the genus *Vibrio*, strain IGJ1.11^T showed a fatty acid profile different from those of its phylogenetically closest species (Table S3).

In conclusion, on the basis of phenotypic, DNA–DNA reassociation, genotypic and phylogenetic characteristics, we suggest that the strains studied represent a novel species of the genus *Vibrio*, for which we propose the name *Vibrio olivae* sp. nov. (type strain IGJ1.11^T).

Description of *Vibrio olivae* sp. nov

Vibrio olivae (o.li′vae. L. gen. n. *olivae* of an olive, referring to the isolation of the strains from olive fermentations).

Cells are Gram-stain-negative, lightly curved rods (1.4 μm × 0.6 μm; Fig S9) and non-motile. Colonies on BHI are circular, convex, with a uniform edge, mucoid and white to cream with a diameter of 2–3 mm after incubation at 37 °C for 24 h. Colonies on TCBS are circular, convex, with a uniform edge, mucoid and yellow with a diameter of 4 mm after incubation at 37 °C for 24 h. Grows in BHI broth (this medium typically contains 0.5% NaCl), not requiring added NaCl for its growth, at pH 5.0–9.0, and in 1–10.0% NaCl. Growth occurs at 15–42 °C, but not at 10 °C or below, nor at 45 °C or above. Optimal growth is achieved aerobically at 30–37 °C, at pH 7.4 in BHI broth medium. Grows anaerobically in BHI-cys medium at 30 °C, but requires at least 1% NaCl for growth. Acid is produced from D-glucose, D-mannitol, sucrose, melibiose, amygdalin, L-arabinose and L-rhamnose, but not from inositol or D-sorbitol. Positive for β-galactosidase and oxidase activities, but negative for catalase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, urease, H_2S production, indole production, and Voges–Proskauer reaction. Positive for hydrolysis of gelatin. Citrate utilization is strain-dependent (IGJ1.11^T is positive, but strains IGJ8.11 and IGJ 10.11 are negative). The predominant cellular fatty acids (>5%) are summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$), $C_{16:0}$ and summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$).

The type strain, IGJ1.11^T (=CECT 8064^T = DSM 25438^T), was isolated from Spanish-style green olive fermentations. The DNA G+C content of the type strain is 52.9 mol%.

Two additional strains of the species are IGJ8.11 and IGJ 10.11, also isolated from green olive fermentations.

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References

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped blast and psi-blast: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.

Baumann, P., Furniss, A. L. & Lee, J. V. (1984). Genus I. *Vibrio* Pacini 1854. In *Bergey's Manual of Systematic Bacteriology*, pp. 518–538. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.

Brenner, D. J., Hickman-Brenner, F. W., Lee, J. V., Steigerwalt, A. G., Fanning, G. R., Hollis, D. G., Farmer, J. J., III, Weaver, R. E., Joseph, S. W. & Seidler, R. J. (1983). *Vibrio furnissii* (formerly aerogenic biogroup of *Vibrio fluvialis*), a new species isolated from human feces and the environment. *J Clin Microbiol* **18**, 816–824.

de Castro, A., Montaño, A., Casado, F.-J., Sanchez, A.-H. & Rejano, L. (2002). Utilization of *Enterococcus casseliflavus* and *Lactobacillus pentosus* as starter culture for Spanish-style green olive fermentation. *Food Microbiol* **19**, 637–644.

Farmer, J. J., III & Hickman-Brenner, F. W. (2006). The genera *Vibrio* and *Photobacterium*. In *The Prokaryotes: a Handbook on the Biology of Bacteria*, pp. 508–563. Edited by M. Dworkin, S. Falkow, E. Rosenberg, K. H. Schleifer & E. Stackebrandt. **vol. 6**, 3rd edn., New York: Springer.

Farmer, J. J., III, Janda, J. M., Brenner, F. W., Cameron, D. N. & Birkhead, K. M. (2005). Genus I. *Vibrio* Pacini 1854, 411^{AL}. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 2, *The Proteobacteria, Part B, The Gammaproteobacteria*,, pp. 494–546. Edited by D. J. Brenner, N. R. Krieg, J. T. Staley & G. M. Garrity. New York: Springer.

Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* **17**, 368–376.

Fernandez, A. G., Garcia, P. G. & Balbuena, M. B. (1995). Olive fermentations. In *Biotechnology: Enzymes, Biomass, Food and Feed*, pp. 593–627. Edited by H.-J. Rehm & G. Reed. **vol. 9**, 2nd edn., Weinheim, Germany: Wiley-VCH.

Fitch, W. M. (1971). Toward defining the course of evolution: minimum change for a specific tree topology. *Syst Zool* **20**, 406–416.

Gao, Z.-M., Xiao, J., Wang, X.-N., Ruan, L.-W., Chen, X. L. & Zhang, Y.-Z. (2012). *Vibrio xiamenensis* sp. nov., a cellulase-producing bacterium isolated from mangrove soil. *Int J Syst Evol Microbiol* **62**, 1958–1962.

Gonzalez, J. M. & Saiz-Jimenez, C. (2002). A fluorimetric method for the estimation of G+C mol% content in microorganisms by thermal denaturation temperature. *Environ Microbiol* **4**, 770–773.

Gonzalez, J. M. & Saiz-Jimenez, C. (2005). A simple fluorimetric method for the estimation of DNA-DNA relatedness between closely related microorganisms by thermal denaturation temperatures. *Extremophiles* **9**, 75–79.

Kalina, G. P., Antonov, A. S., Turova, T. P. & Grafova, T. I. (1984). *Allomonas enterica* gen. nov., sp. nov., deoxyribonucleic acid homology between *Allomonas* and some other members of the *Vibrionaceae*. *Int J Syst Bacteriol* **34**, 150–154.

Kim, O. S., Cho, Y. J., Lee, K., Yoon, S. H., Kim, M., Na, H., Park, S. C., Jeon, Y. S., Lee, J. H. & other authors (2012). Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int J Syst Evol Microbiol* **62**, 716–721.

Kim, M., Oh, H. S., Park, S. C. & Chun, J. (2014). Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes. *Int J Syst Evol Microbiol* **64**, 346–351.

Lane, D. J. (1991). 16/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. Chichester: Wiley.

Lazo, G. R., Roffey, R. & Gabriel, D. W. (1987). Pathovars of *Xanthomonas campestris* are distinguishable by restriction fragment-length polymorphism. *Int J Syst Bacteriol* **37**, 214–221.

Lucena-Padrós, H., Caballero-Guerrero, B., Maldonado-Barragan, A. & Ruiz-Barba, J. L. (2014). Microbial diversity and dynamics of Spanish-style green table-olive fermentations in large manufacturing companies through culture-dependent techniques. *Food Microbiol* **42**, 154–165.

Maldonado-Barragan, A., Caballero-Guerrero, B., Lucena-Padrós, H. & Ruiz-Barba, J. L. (2013). Induction of bacteriocin production by coculture is widespread among plantaricin-producing *Lactobacillus plantarum* strains with different regulatory operons. *Food Microbiol* **33**, 40–47.

Meier-Kolthoff, J. P., Auch, A. F., Klenk, H. P. & Göker, M. (2013). Genome sequence-based species delimitation with confidence intervals and improved distance functions. *BMC Bioinformatics* **14**, 60.

Myers, E. W. & Miller, W. (1988). Optimal alignments in linear space. *Comput Appl Biosci* **4**, 11–17.

Noguerola, I. & Blanch, A. R. (2008). Identification of *Vibrio* spp. with a set of dichotomous keys. *J Appl Microbiol* **105**, 175–185.

Rejano, L., Montaño, A., Casado, F. J., Sanchez, A. H. & de Castro, A. (2010). Table olives: varieties and variations. In *Olives and Olive Oil in Health and Disease Prevention*, pp. 5–15. Edited by V. R. Preedy & R. R. Watson. Oxford: Academic Press.

Ruiz-Barba, J. L. & Jiménez-Diaz, R. (2012). A novel *Lactobacillus pentosus*-paired starter culture for Spanish-style green olive fermentation. *Food Microbiol* **30**, 253–259.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Sanchez, A. H., Rejano, L., Montaño, A. & de Castro, A. (2001). Utilization at high pH of starter cultures of lactobacilli for Spanish-style green olive fermentation. *Int J Food Microbiol* **67**, 115–122.

Sasser, M. (1990). Identification of bacteria by gas chromatography of cellular fatty acids, MIDI Technical Note 101. Newark, DE: MIDI Inc.

Sawabe, T., Kita-Tsukamoto, K. & Thompson, F. L. (2007). Inferring the evolutionary history of vibrios by means of multilocus sequence analysis. *J Bacteriol* **189**, 7932–7936.

Sawabe, T., Ogura, Y., Matsumura, Y., Feng, G., Amin, A. R., Mino, S., Nakagawa, S., Sawabe, T., Kumar, R. & other authors (2013). Updating the *Vibrio* clades defined by multilocus sequence phylogeny: proposal of eight new clades, and the description of *Vibrio tritonius* sp. nov. *Front Microbiol* **4**, 414.

Stackebrandt, E. & Ebers, J. (2006). Taxonomic parameters revisited: tarnished gold standards. *Microbiol Today* **33**, 152–155.

Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011). mega5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28**, 2731–2739.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). clustal w: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Thompson, F. L., Iida, T. & Swings, J. (2004). Biodiversity of vibrios. *Microbiol Mol Biol Rev* **68**, 403–431.

Thompson, F. L., Gevers, D., Thompson, C. C., Dawyndt, P., Naser, S., Hoste, B., Munn, C. B. & Swings, J. (2005). Phylogeny and molecular identification of vibrios on the basis of multilocus sequence analysis. *Appl Environ Microbiol* **71**, 5107–5115.

Veyrat, A., Miralles, M. C. & Pérez-Martinez, G. (1999). A fast method for monitoring the colonization rate of lactobacilli in a meat model system. *J Appl Microbiol* **87**, 49–61.

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.

Fig S1. RAPD profiles and dendrogram (UPGMA) representing genetic relationships among *Vibrio olivae* isolates based on genetic similarity matrix, calculated by using the pair-wise Pearson's correlation coefficient. The scale indicates the similarity level. The genotype indicates the grouping of the isolates according to their RAPD profile. The number of the fermenter where the isolated were recovered are indicated. Molecular weight marker (1Kb Plus, Invitrogen) used as a reference is shown on top of the figure.

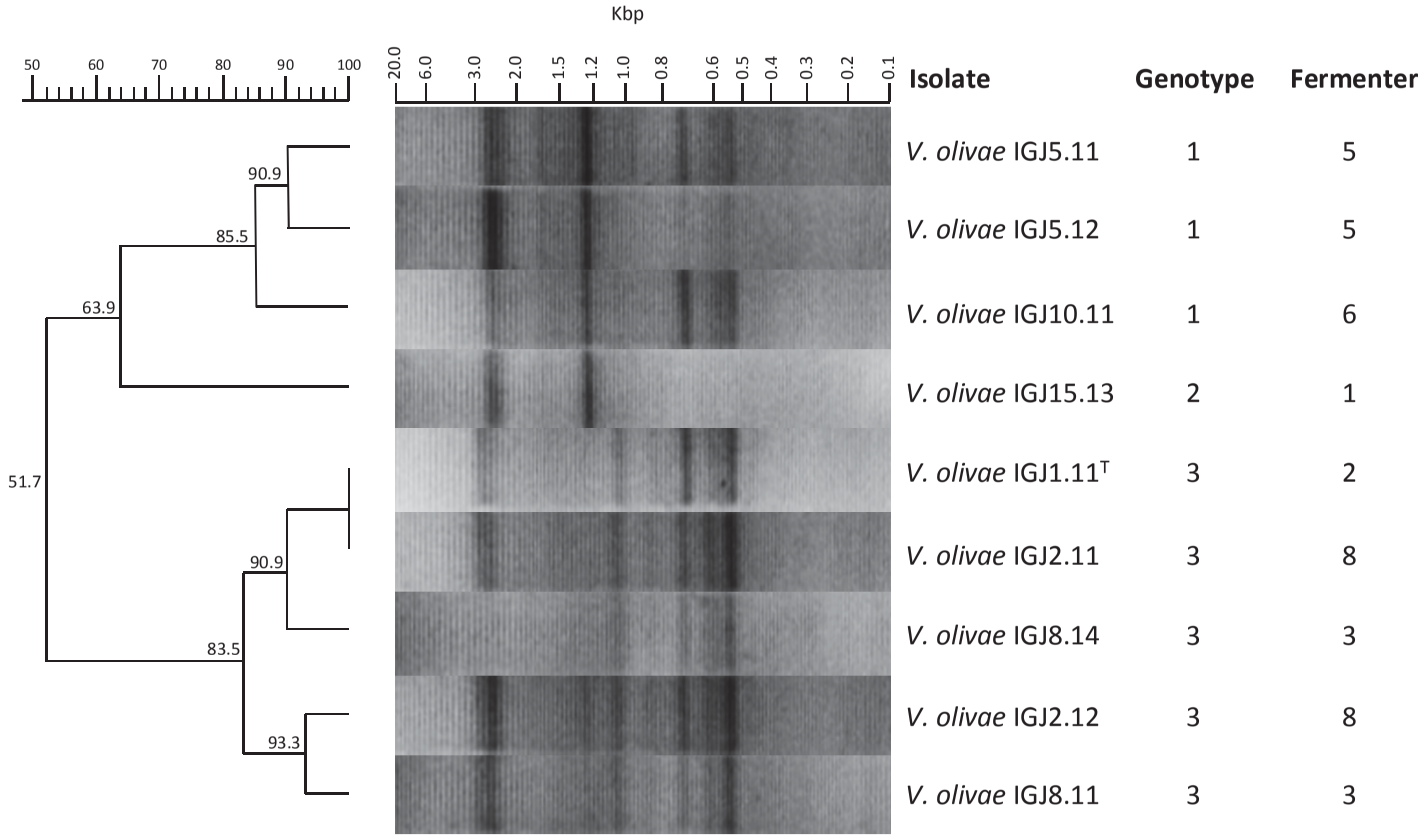

Fig S2. Phylogenetic tree based on almost complete 16S rRNA gene sequences using the maximum likelihood method. Numbers on the tree indicate bootstrap values calculated for 1000 subsets for branch-points greater than 50%. *Photobacterium damselae* was used as an outgroup. GenBank sequence accession numbers are given in parentheses. Bar, 5% estimated divergence. Bar, 0.005 nucleotide changes per nucleotide position. For clarity, only the isolate IGJ1.11^T is shown.

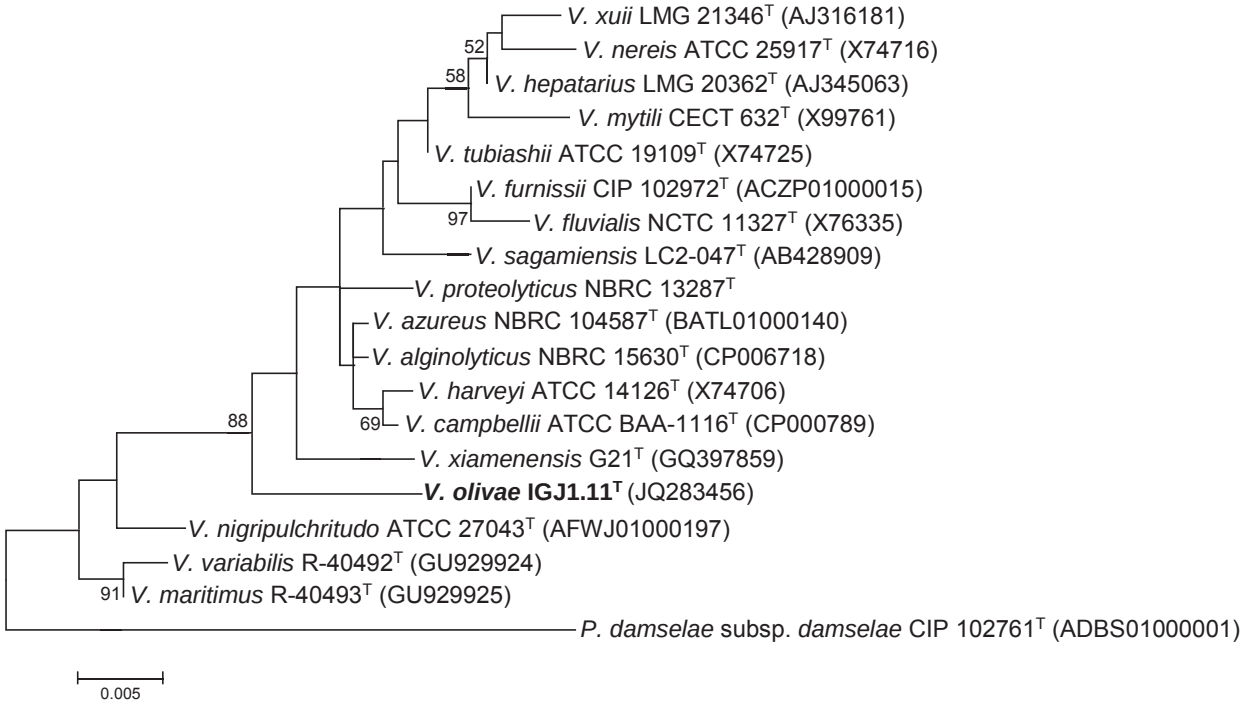

Fig S3. Maximum-parsimony phylogenetic tree showing the position of *Vibrio olivae* IGJ1.11^{T} based on almost complete 16S rRNA gene sequences. Numbers on the tree indicate bootstrap values calculated for 1000 subsets for branch-points greater than 50%. *Photobacterium damselae* was used as an outgroup. GenBank sequence accession numbers are given in parentheses. Bar, 5% estimated sequence divergence.

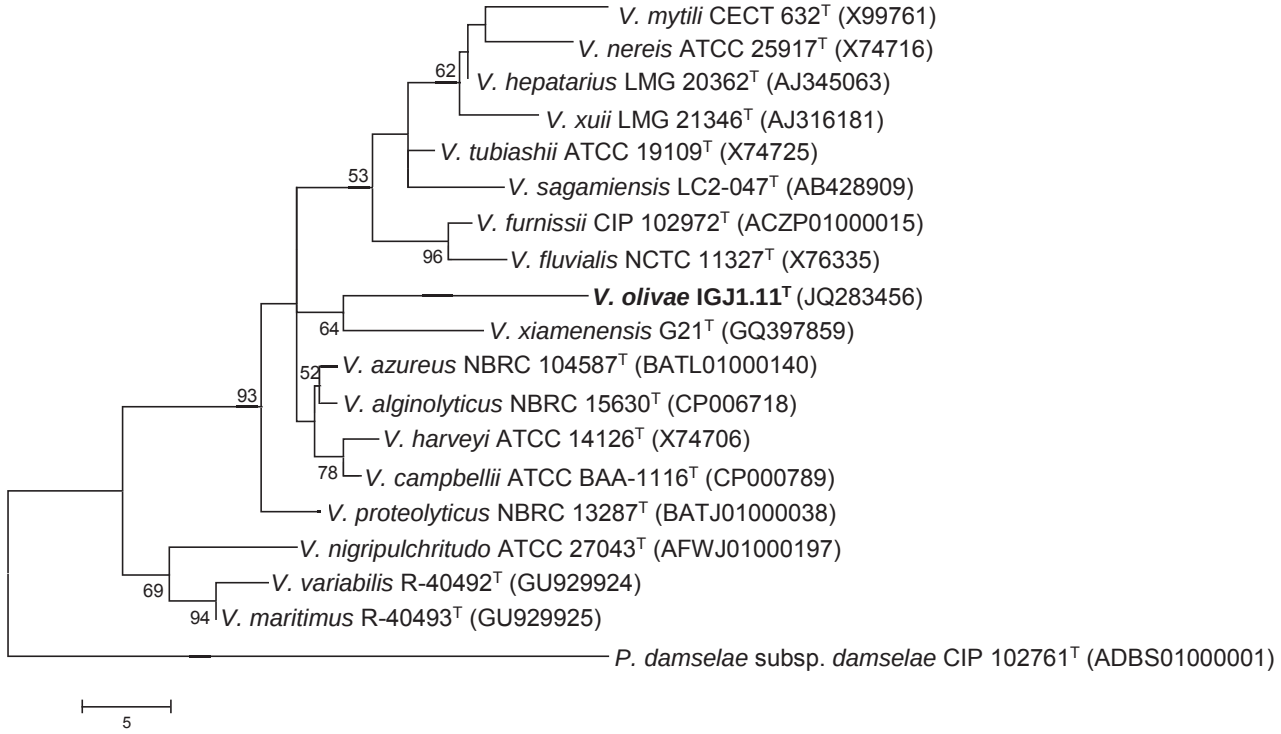

Fig S4. Neighbour-joining phylogenetic tree showing the position of *Vibrio olivae* IGJ1.11^{T} based on *pyrH* gene sequences (406 bp). Bootstrap values (>50 %) shown are based on 1000 repetitions. Bar, 2% estimated sequence divergence.

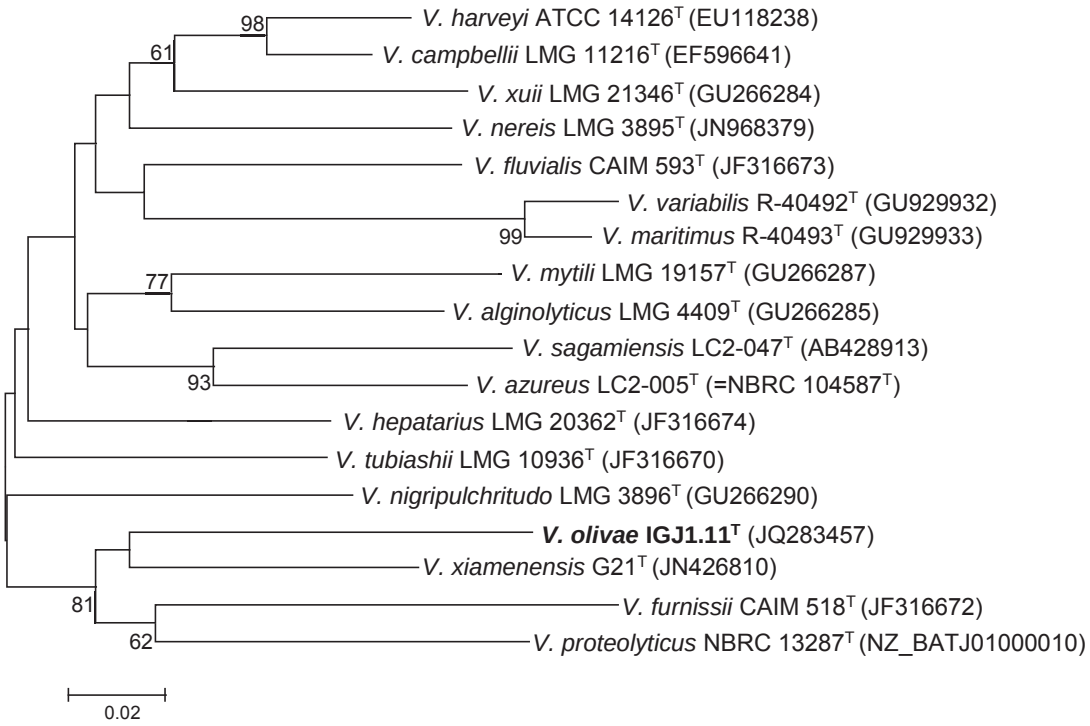

Fig S5. Neighbour-joining phylogenetic tree showing the position of *Vibrio olivae* IGJ1.11^T based on *recA* gene sequences (498 bp). Bootstrap values (>50 %) shown are based on 1000 repetitions. Bar, 2% estimated sequence divergence.

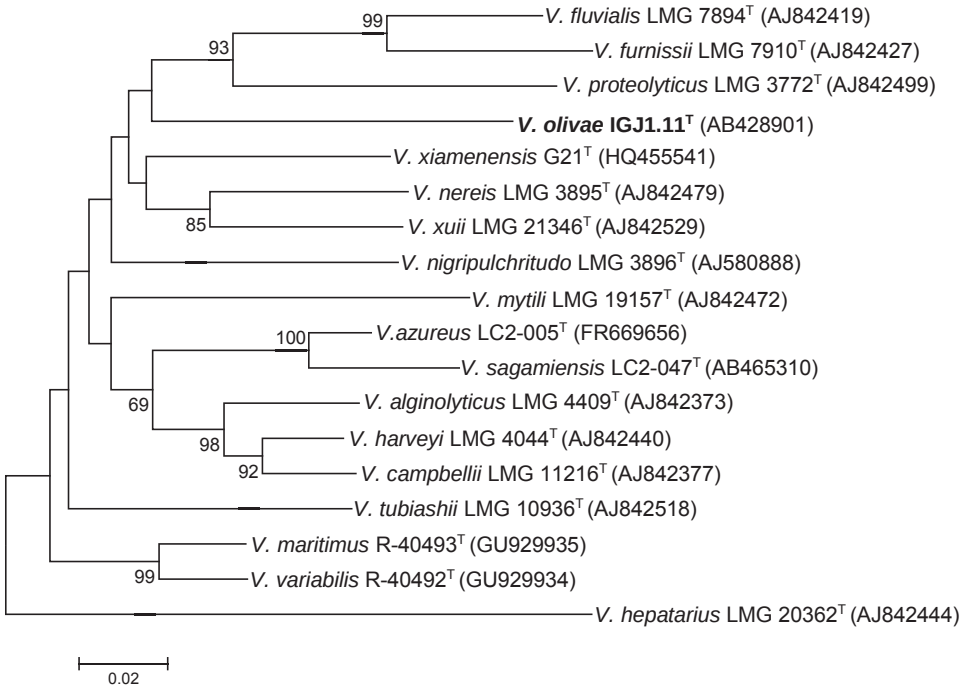

Fig S6. Neighbour-joining phylogenetic tree showing the position of *Vibrio olivae* IGJ1.11^{T} based on *rpoA* gene sequences (790 bp). Bootstrap values (>50 %) shown are based on 1000 repetitions. Bar, 1% estimated sequence divergence.

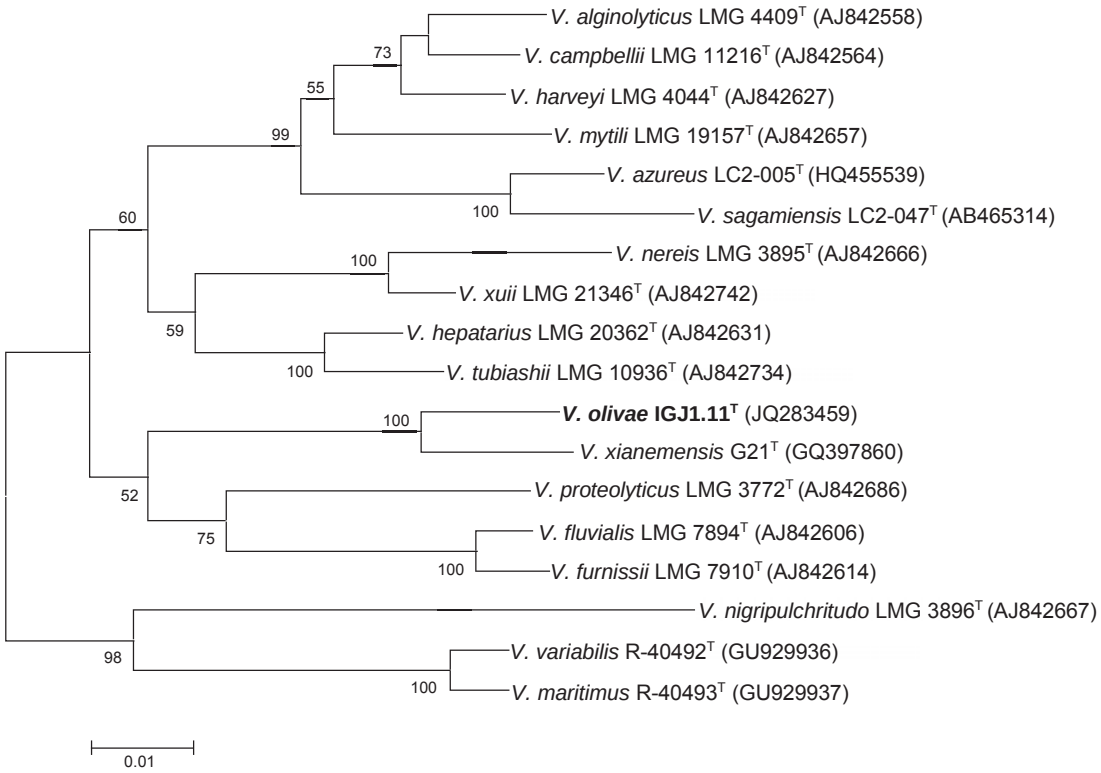

Fig S7. Neighbour-joining phylogenetic tree showing the position of *Vibrio olivae* IGJ1.11^{T} based on *gyrB* gene sequences (595 bp). Bootstrap values (>50 %) shown are based on 1000 repetitions. Bar, 2% estimated sequence divergence.

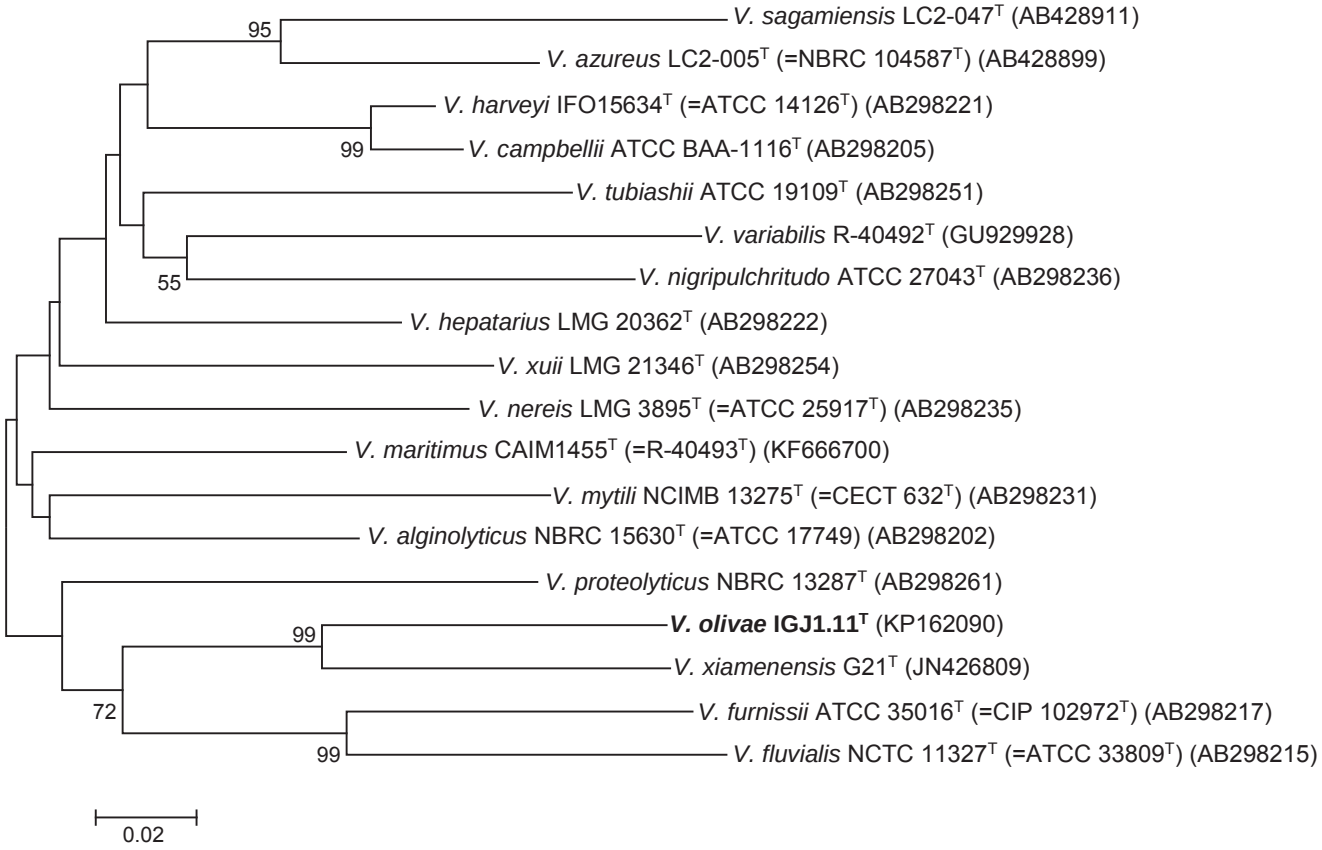

Fig S8. Neighbour-joining phylogenetic tree showing the position of *Vibrio olivae* IGJ1.11^{T} based on *mreB* gene sequences (538 bp). Bootstrap values (>50 %) shown are based on 1000 repetitions. Bar, 2% estimated sequence divergence.

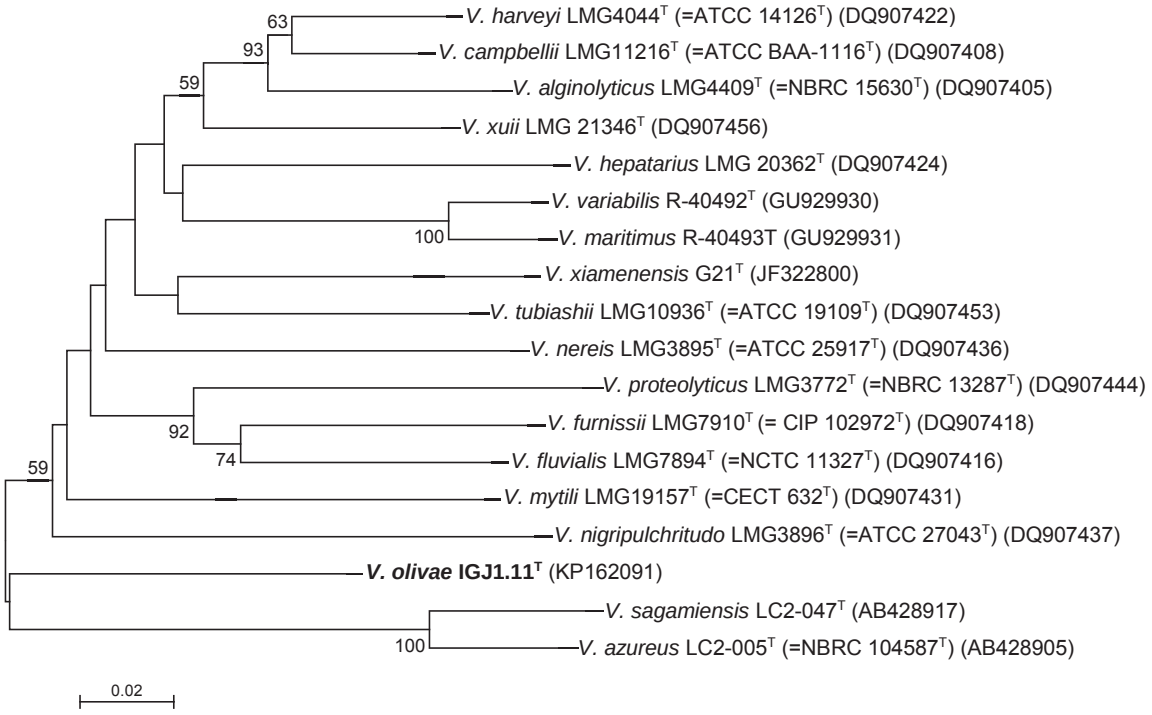

Fig S9. Phase-contrast photomicrographs of *Vibrio olivae* IGJ1.11^{T} from cultures grown for 14 h at 30 ºC in Marine Agar.

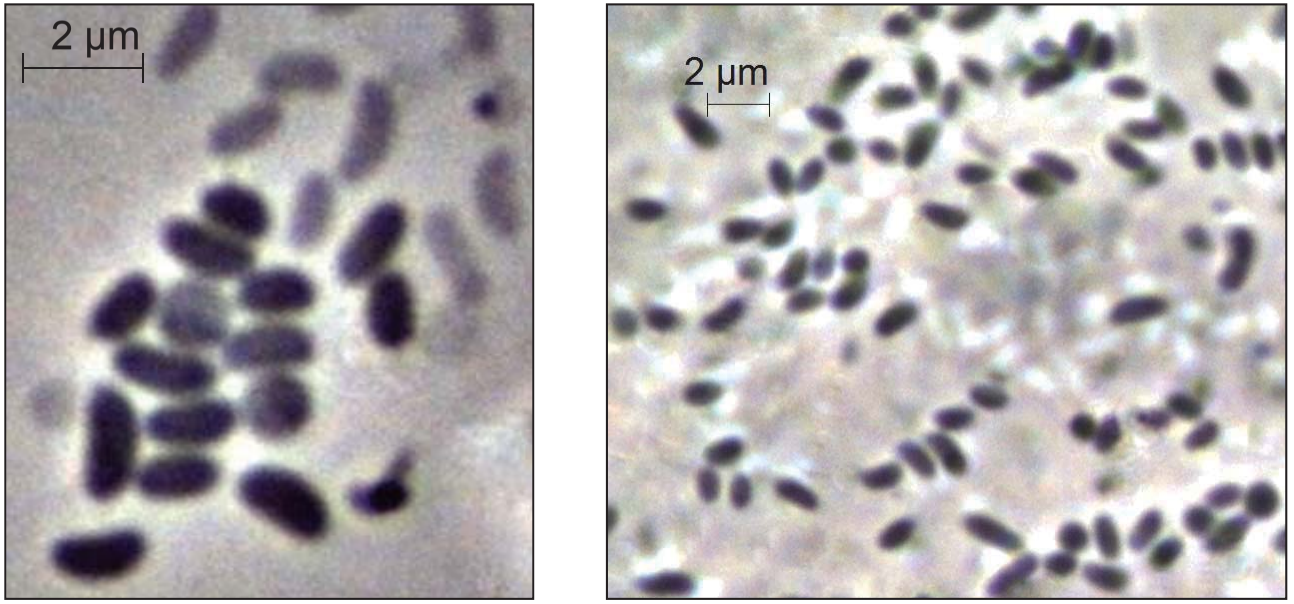

Table S1. GenBank accession numbers of the housekeeping genes sequences of *Vibrio olivae* sp. nov. and the type strains of the phylogenetically related *Vibrio* species used in this study.

V. olivae IGJ1.11^{T}	KP162090	KP162091	JQ283457	JQ283458	JQ283459
V. xiamenensis G21^{T}	JN426809	JF322800	JN426810	HQ455541	GQ397860
V. furnissii CIP 102972^{T} (=CAIM 518^{T}=LMG 7910^{T}=ATCC 35016^{T})	AB298217	DQ907418	JF316672	AJ842427	AJ842614
V. fluvialis NCTC 11327^{T} (=CAIM 593^{T}=LMG 7894^{T}=NCTC 11327^{T})	AB298215	DQ907416	JF316673	AJ842419	AJ842606
V. variabilis R-40492^{T}	GU929928	GU929930	GU929932	GU929934	GU929936
V. hepatarius LMG 20362^{T}	AB298222	DQ907424	JF316674	AJ842444	AJ842631
V. mytili CECT 632^{T} (=LMG 19157^{T}=NCIMB 13275^{T})	AB298231	DQ907431	GU266287	AJ842472	AJ842657
V. maritimus R-40493^{T} (=CAIM1455^{T})	KF666700	GU929931	GU929933	GU929935	GU929937
V. tubiashii ATCC 19109^{T} (=LMG 10936^{T})	AB298251	DQ907453	JF316670	AJ842518	AJ842734
V. sagamiensis LC2-047^{T}	AB428911	AB428917	AB428913	AB465310	AB465314
V. proteolyticus NBRC 13287^{T} (=LMG 3772^{T})	AB298261	DQ907444	BATJ01000010	AJ842499	AJ842686
V. nigripulchritudo ATCC 27043^{T} (=LMG 3896^{T})	AB298236	DQ907437	GU266290	AJ580888	AJ842667
V. xuii LMG 21346^{T}	AB298254	DQ907456	GU266284	AJ842529	AJ842742
V. azureus LC2-005^{T} (=NBRC 104587^{T})	AB428899	AB428905	AB428901	FR669656	HQ455539
V. nereis ATCC 25917^{T} (=LMG 3895^{T})	AB298235	DQ907436	JN968379	AJ842479	AJ842666
V. harveyi ATCC 14126^{T} (=LMG 4044^{T}=IFO15634^{T})	AB298221	DQ907422	EU118238	AJ842440	AJ842627
V. alginolyticus NBRC 15630^{T} (=LMG 4409^{T})	AB298202	DQ907405	GU266285	AJ842373	AJ842558
V. campbellii ATCC BAA-1116^{T} (=LMG 11216^{T})	AB298205	DQ907408	EF596641	AJ842377	AJ842564

Table S2. Sequence similarity values (%) between the *gyrB*, *mreB*, *pyrH*, *recA*, *rpoA*, 16S rRNA and concatenated (*recA* + *rpoA* + *pyrH* + *gyrB* + *mreB*; total length, 2827 bp) gene sequences of *Vibrio olivae* IGJ1.11^T and the type strains of phylogenetically related *Vibrio* species. The most similar values for each gene is shown in bold figures.

Strains	*gyrB*	*mreB*	*pyrH*	*recA*	*rpoA*	16SrRNA	Concatenated
V. xiamenensis G21^T	**87.6**	84.8	**86.7**	85.7	**97.3**	**98.0**	**89.7**
V. furnissii CIP 102972^T (=CAIM 518^T=LMG 7910^T=ATCC 35016^T)	79.4	82.4	82.0	82.9	92.3	97.6	85.1
V. fluvialis NCTC 11327^T (=CAIM 593^T=LMG 7894^T=NCTC 11327^T)	78.4	82.6	83.5	83.8	92.7	97.4	85.6
V. variabilis R-40492 ^{T}	77.3	81.7	82.4	85.8	89.4	97.2	84.3
V. hepatarius LMG 20362 ^{T}	79.2	82.7	81.9	77.0	92.8	97.1	84.3
V. mytili CECT 632 ^{T} (=LMG 19157 ^{T}=NCIMB 13275^T)	79.1	82.4	82.8	84.3	91.2	96.9	84.7
V. maritimus R-40493 ^{T} (=CAIM1455^T)	80.4	81.4	81.3	**86.5**	89.2	96.9	85.0
V. tubiashii ATCC 19109 ^{T} (=LMG 10936^T)	80.4	83.2	83.2	82.4	93.1	96.9	85.8
V. sagamiensis LC2-047 ^{T}	79.2	79.3	82.2	84.1	90.4	96.9	84.2
V. proteolyticus NBRC 13287 ^{T} (=LMG 3772^T)	80.1	81.3	84.7	83.4	93.3	96.8	85.8
V. nigripulchritudo ATCC 27043 ^{T} (=LMG 3896^T)	79.2	81.8	83.2	85.0	89.4	96.8	85.1
V. xuii LMG 21346 ^{T}	82.8	**84.9**	82.4	83.4	93.4	96.7	86.4
V. azureus LC2-005^T (=NBRC 104587 ^{T})	79.0	81.4	83.1	85.5	91.5	96.6	85.3
V. nereis ATCC 25917 ^{T} (=LMG 3895^T)	82.3	83.3	83.7	84.5	92.5	96.6	86.3
V. harveyi ATCC 14126 ^{T} (=LMG 4044^T=IFO15634^T)	79.0	83.2	83.4	84.4	90.9	96.6	85.4
V. alginolyticus NBRC 15630 ^{T} (=LMG 4409^T)	81.8	82.8	83.1	84.5	92.2	96.5	86.0
V. campbellii ATCC BAA-1116 ^{T} (=LMG 11216^T)	78.3	83.6	84.4	83.9	91.9	96.5	85.5

Table S3. Cellular fatty acid contents of *V. olivae* IGJ1.11^T. [1]Values are percentages of total fatty acids. Fatty acids that represented <1 % are not shown. *Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 2, $C_{14:0}$ 3OH and/or iso-$C_{16:1}$ I and/or $C_{12:0}$ aldehyde and/or unknown; summed feature 3, $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$; summed feature 8, $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$.

Fatty acid	%[1]
$C_{12:0}$	4.2
$C_{12:0}$ 3-OH	4.1
$C_{14:0}$	4.4
$C_{16:0}$	18.3
iso-$C_{16:0}$	2.1
$C_{17:0}$	1.2
$C_{17:1}$ *ω6c*	–
$C_{17:1}$ *ω8c*	1.4
$C_{18:0}$	1.0
iso-$C_{18:0}$	1.2
11-Methyl $C_{18:1}$ *ω7c*	1.1
Summed feature 2*	3.6
Summed feature 3*	36.4
Summed feature 8*	17.9

4.7 Diversity and enumeration of halophilic and alkaliphilic bacteria in Spanish-style green table-olive fermentations.

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Diversity and enumeration of halophilic and alkaliphilic bacteria in Spanish-style green table-olive fermentations

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ABSTRACT

The presence and enumeration of halophilic and alkaliphilic bacteria in Spanish-style table-olive fermentations was studied. Twenty 10-tonne fermenters at two large manufacturing companies in Spain, previously studied through both culture dependent and independent (PCR-DGGE) methodologies, were selected. Virtually all this microbiota was isolated during the initial fermentation stage. A total of 203 isolates were obtained and identified based on 16S rRNA gene sequences. They belonged to 13 bacterial species, included in 11 genera. It was noticeable the abundance of halophilic and alkaliphilic lactic acid bacteria (HALAB). These HALAB belonged to the three genera of this group: *Alkalibacterium, Marinilactibacillus* and *Halolactibacillus*. Ten bacterial species were isolated for the first time from table olive fermentations, including the genera *Amphibacillus, Natronobacillus, Catenococcus* and *Streptohalobacillus*. The isolates were genotyped through RAPD and clustered in a dendrogram where 65 distinct strains were identified. Biodiversity indexes found statistically significant differences between both *patios* regarding genotype richness, diversity and dominance. However, Jaccard similarity index suggested that the halophilic/alkaliphilic microbiota in both *patios* was more similar than the overall microbiota at the initial fermentation stage. Thus, up to 7 genotypes of 6 different species were shared, suggesting adaptation of some strains to this fermentation stage. Morisita-Horn similarity index indicated a high level of codominance of the same species in both *patios*. Halophilic and alkaliphilic bacteria, especially HALAB, appeared to be part of the characteristic microbiota at the initial stage of this table-olive fermentation, and they could contribute to the conditioning of the fermenting brines in readiness for growth of common lactic acid bacteria.

1. Introduction

Table olives represent a typical component of the Mediterranean diet and their production has a great economical and social impact in these countries (IOOC, 2014). This vegetable fermentation can be elaborated by a wide variety of traditional procedures, although the three most common industrial processing methods for the international trade market are Spanish-style green olives, California-style oxidised black olives and Greek-style natural black olives (Rejano et al., 2010). In Spain, the world leading table olive producing country, Spanish-style green olives is the most popular preparation. It is characterised by an initial treatment of the green fruits with a dilute (2.5–3.0%, w/v) sodium hydroxide solution ("lye") as a fast de-bittering procedure, involving the hydrolysis of oleuropein, followed by one or more water washing step to remove the excess of lye (De Castro et al., 2002; Aponte et al., 2012). Finally, the treated fruits are placed into 10,000 to 15,000-kg glass-fibre containers and covered with a brine of a salt concentration ranging 10–12% (w/v). These conditions allow a multistep spontaneous fermentation where at least three distinct stages can be defined (Garrido Fernández et al., 1995). This fermentation is carried out by strains of the species *Lactobacillus pentosus*, although other lactic acid bacteria (LAB) can be also involved (De Castro et al., 2002; Lucena-Padrós et al., 2014b; Rejano et al., 2010; Ruiz-Barba and Jiménez-Díaz, 2012). However, during the first fermentation stage, lasting 3–10 days, a heterogeneous microbiota is usually present which takes advantage of the high salt and pH values of these brines at that moment (De Castro et al., 2002). Actually, several authors have isolated (Abdelkafi et al., 2006; De Castro et al., 2002; Ntougias and Russel, 2000; Quesada et al., 2007) or detected

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through culture-independent techniques (Abriouel et al., 2011; Cocolin et al., 2013) halophilic and/or alkaliphilic bacteria from table olive fermentations, including the effluents derived from their preparation, as it is the case of *Alkalibacterium olivoapovliticus* (Ntougias and Russell, 2001).

Recently, several comprehensive studies on the microbial ecology associated to Spanish-style green table-olive fermentations at the industrial level have been reported (Lucena-Padrós et al., 2014b, 2014c, 2015b). Both culture-dependent and independent techniques were used on the same samples in an attempt to update our knowledge on this fermentation. When PCR-DGGE was used to examine this fermentations, results revealed that several halophilic and alkaliphilic bacterial species, not isolated before from table-olive fermentations, could play a relevant role in Spanish-style olive fermentations, as they were widespread in the fermenters and fermentation yards (*patios*) under study (Lucena-Padrós et al., 2015b). It was remarkable the presence of halophilic and alkaliphilic LAB (HALAB), a bacterial group which includes the genera *Alkalibacterium, Halolactibacillus* and *Marinilactibacillus* (Ntougias, 2012). As in the cognate, previous culture-dependent studies no selective culture medium was used to specifically examine the presence of this group of bacteria, only a few halophilic and/or alkaliphilic bacteria were isolated on that occasion, including *Aerococcus viridans/urinaeequi, Enterococcus olivae* (previously identified as *Enterococcus saccharolyticus*), *Enterococcus casseliflavus* and *Vibrio olivae* (previously identified as *Vibrio furnisii/fluvialis*). The aim of this study is to corroborate the presence of halophilic/alkalophilic bacteria, previously detected through PCR-DGGE in Spanish-style green table-olive fermentations, as well to assess their presence through the fermentation time and estimate their possible role. For this, we have used the same fermenting-brine samples which were used before in the mentioned PCR-DGGE study and specific selective culture media designed to rescue such microbiota.

2. Materials and methods

2.1. Origin of the samples and sampling strategy

Samples of Spanish-style green-olive fermenting brines were obtained from 20 10-tonne fermenters at two large (4000–8000 t olives handled per season) manufacturing companies in the province of Sevilla, southern Spain. At each company, fermentation was followed in ten fermenters, each of them of a total capacity of 10 tonnes of olives and 5500–6000 L of brine, made in polyester and glass fibre. These fermenters were located outdoor, buried in the ground of the respective fermentation yards, what it is traditionally called in Spain a "*patio*". The traditional Spanish-style procedure to prepare green olives (Rejano et al., 2010) was followed, and a detailed description was made previously (Lucena-Padrós et al., 2014b). Olives were all of the Manzanilla variety and no starter culture was used. Three consecutive 50-ml samples were taken from approximately the geometric centres of each fermenter at approximately monthly intervals, in coincidence with the initial, middle and final stages of the green table-olive fermentation. More specifically, fermentation had taken place for 1 to 14 (first two weeks), 35 to 48 (5th to 7th week), and 69 to 82 (10th to 12th week) days after brining, for the initial, middle and final sampling points, respectively. Samples were stored at −80 °C in 20% (v/v) glycerol until analysed. These same fermenting brines had been analysed previously through culture-dependent (Lucena-Padrós et al., 2014b, 2014c) and independent (PCR-DGGE; Lucena-Padrós et al., 2015b) techniques. Fermentation time, pH and NaCl concentration of these samples are shown in Table S1.

2.2. Isolation and enumeration of microorganisms

Aliquots of brine samples were defrost at room temperature. After vigorous vortexing, serial 10-fold dilutions were performed in 0.1% (w/v) peptone water and plated in duplicates onto agar plates of culture media. Two different alkaline and high salt-content media were used as follows: a) RCMAS, consisting of Reinforced Clostridial Medium (RCM; Biokar Diagnostics) containing 100 mM $NaHCO_3/Na_2CO_3$ buffer (pH 10) supplemented with 7% (w/v) NaCl; b) GYECS, based on GYEC medium (Ntougias and Russell, 2001) and composed of 1% (w/v) glucose (Sigma), 0.5% (w/v) yeast extract (Oxoid), 7% NaCl (w/v), 0.1% (w/v) L-cysteine (AppliChem), and a buffer (100 mM Na_2CO_3/1 mM K_2HPO_4, pH10.5) containing 0.1% (w/v) NH_4SO_4 plus 0.1 mM $MgSO_4{}^*7H_2O$. Agar was added to the broth media at 1.5% (w/v). Seeded plates were incubated anaerobically at 30 °C for three days, using a DG250 Anaerobic Workstation (Don Whitley Scientific Ltd., Shipley, West Yorkshire, UK), with a gas mixture consisting of 10% H_2–10% CO_2–80% N_2.

Isolate colonies appearing in the plates were classified attending to their shape, colour, texture, size, etc., as well to their cell morphology, cell arrangement, motility and spore forming ability as observed under a phase-contrast microscope (Olympus Optical Co., Tokyo, Japan). For further studies, a single colony of each different morphotype identified in both culture media at each sampling point was selected from plates with low counts and purified by repeated subculturing. For long-term storage, purified isolates were preserved at −80 °C in the culture medium they were initially isolated containing glycerol (20% v/v). All isolates were subjected to genotyping through the RAPD technique as described below.

2.3. Molecular identification techniques

Total DNA of the isolates was extracted directly from colonies by the rapid chloroform method described by Ruiz-Barba et al. (2005). Genotyping and molecular identification of the isolates was carried out as described below.

2.3.1. Genotyping through RAPD

Genotyping was carried out by RAPD using the primer OPL5 (5′-ACGCAGGCAC-3′) as described by Maldonado-Barragán et al. (2013). Amplification products were electrophoretically resolved through 2% (w/v) agarose gels (SeaKem, Biowhittaker Molecular Applications, USA) in 1x TAE buffer, stained with ethidium bromide (0.5 μg/ml), visualized under UV light and digitally recorded. DNA molecular weight marker 1-kb Plus DNA Ladder (Invitrogen) was used as size standard and as a normalization reference. Reference strains *E. olivae* IGG16.11^T (Lucena-Padrós et al., 2014a and 2014c; previously identified as *E. saccharolyticus* in Lucena-Padrós et al., 2014b) and *V. olivae* IGJ1.11v^T (Lucena-Padrós et al., 2015a; previously identified as *Vibrio furnissii/fluvialis* J1.11v in Lucena-Padrós et al., 2014b and 2014c) were included in the cluster analysis of the RAPD profiles in order to produce an improved distinction among species. The resulting RAPD profiles were normalized and analysed for clustering with the Bionumerics 7.0 software package (Applied Maths, Sint-Martens-Latem, Belgium). Only bands representing amplicons between 150 and 5000 bp in size were included in the analysis. Similarity dendrograms were constructed by the UPGMA clustering method, using the band-based Dice similarity coefficient. The quality of the cluster analysis was verified by calculating the cophenetic correlation value (in percentage) for each dendrogram, using the BioNumerics 7.0 software. Interpretation of values obtained for the similarity coefficients was as follows: 1.0, genetically indistinguishable isolates; 0.99 to 0.80, closely related isolates that are highly similar but not identical, which could be considered the same strain; 0.79 to 0.50, related isolates;

<0.50, unrelated isolates (Tenover et al., 1995; Soll, 2000). As a control, reproducibility of the PCR fingerprinting experiments was verified with a reduced number of strains.

2.3.2. Molecular identification through 16S rRNA gene sequence analysis

Bacterial isolates were identified to the genus and/or species level by PCR sequencing of a *ca.* 500-bp fragment of the 16S rRNA gene, using the primer pair plb16/mlb16 (Kullen et al., 2000). PCR conditions were as described by Delgado et al. (2008). Briefly: initial denaturation at 96 °C for 30 s, followed by 30 cycles of denaturation at 96 °C for 30 s, annealing at 50 °C for 30 s, and polymerisation at 72 °C for 45 s, plus a final polymerisation step at 72 °C for 4 min. MyTaq DNA polymerase (Bioline, London, UK) was used according to the manufacturer instructions. The resulting amplicons were purified using a Nucleospin Extract II kit (Macherey–Nagel, Düren, Germany) and sequenced at Newbiotechnic S.A. (Bollullos de la Mitación, Spain). The resulting sequences were used to search for similarities using the BLASTN program on the basis of 16S rRNA gene sequence data obtained (Altschul et al., 1997) against the database containing type strains with updated validly published prokaryotic names, by using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim et al., 2012). The identities of the representative isolates were determined on the basis of the highest scores (typically ≥ 98.5%). When necessary, e.g. when the partial sequence of 16S rRNA gene was not sufficient for a clear-cut identification, the complete 16S rRNA gene was PCR amplified (*ca.* 1400 bp) with the primer pair 7for (5′-AGAGTTTGATYMTGGCTCAG-3′) and 1510r (5′-TACGGYTACCTTGTTACGACTT-3′) (Lane, 1991), and the resulting amplicon sequenced and analysed as described above. In these cases, the almost full-length 16S rRNA gene sequences were assembled using the Seqman software version 5.01 (DNASTAR, USA). Finally, sequences (*ca.* 500 or 1400-bp-long 16S rRNA gene sequences) were aligned with CLUSTAL W (Thompson et al., 1994), checked manually and grouped into operational taxonomic units (OTUs) or phylotypes using a ≥ 98.5% similarity threshold. A representative 16S rRNA gene sequence from each OTU was then archived in the GenBank database.

2.3.3. Phylogenetic analysis of partial 16S rRNA gene sequences

Phylogenetic trees based on the partial 16S rRNA gene sequences were constructed using MEGA version 5.0 (Tamura et al., 2011) with the neighbour-joining method (Saito and Nei, 1987) and 1000 replicates of bootstrap analysis. Phylogenetic analyses were restricted to nucleotide positions that could be unambiguously aligned in all representative sequences of each OTU selected together with that of their closest relatives, as downloaded from databases.

2.4. Biodiversity analyses

Biodiversity of the overall microbial load was evaluated with Margalef's index of genotypes richness (R), Shannon–Weaver's index of diversity (H′) and Simpson's index of dominance (D), calculated as proposed by Ventorino et al. (2007) for each fermenter. Comparisons of mean values of biodiversity indexes between *patios* were done by t-Student's tests. Levene tests were used to check for homogeneity of the variance, while Shapiro–Wilk test was used to check for normality. A probability value of $P < 0.05$ was regarded to be statistically significant. These analyses were performed using the SPSS 21.0 statistical software (SPSS Inc., Chicago, USA). Venn diagram was drawn using the Venn Diagram Plotter (Pacific Northwest National Laboratory, Richland, WA, U.S.A.). The number of halophilic/alkaliphilic species shared between *patios* along the fermentation was estimated using Jaccard qualitative similarity index (Magurran, 1988). Morisita-Horn similarity index (Magurran, 1988) was also calculated as a quantitative index weighing shared species by their relative genotype diversity using the following formula:

$$C_{MH} = 2\Sigma(an_i * bn_i)/(Da + Db)(aN)(bN)$$

where an_i and bn_i is the total number of different genotypes in the ith species in *patio* 1 and *patio* 2, respectively; Da and Db is the Simpson's index of dominance calculated as proposed by Ventorino et al. (2007) in *patio* 1 and *patio* 2, respectively; aN and bN is the total number of genotypes in *patio* 1 and *patio* 2, respectively.

2.5. Statistical analyses

Total counts of microorganisms were expressed as the mean values of colony forming units (CFU) per millilitre of brine based on duplicate analyses made to each sample. The resulting values were transformed to logarithmic values before statistical analyses were performed. To compare paired population densities quantified on RCMAS and GYECS media, Wilcoxon's signed-ranks test for two groups was applied. The Spearman rank coefficient of correlation was also calculated. Finally, to determine statistically significant differences between the microbial counts in both *patios* at each sampling point and for each culture media (RCMAS and GYECS) U Mann–Whitney test was used. These analyses were performed using the SPSS 21.0 statistical software (SPSS Inc., Chicago, USA).

3. Results

3.1. Total counts and evolution of halophilic and alkaliphilic bacteria in the fermenting olive brines

Total counts of the microbial population isolated on RCMAS and GYECS culture media during Spanish-style green olive fermentations are shown in Table 1 and Fig. S1. At each sampling point, counts were very similar in both culture media, being Pearson's coefficient 0.96, while no significant differences were found by the Wilcoxon test when this statistic was applicable. As expected, the highest counts were obtained at the initial fermentation stage, were pH values and salt concentrations (Table S1) were still high in the fermenting brines. As fermentation progressed, and pH became more acidic, this microbiota decreased dramatically, especially in *patio* 1 (Table 1 and Fig. S1). No statistical differences could be found between the results obtained in both culture media, i.e. RCMAS and GYECS, at any sampling point. However, statistically significant differences could be found between both *patios* at the initial fermentation stage, being the halophilic/alkaliphilic microbiota more abundant in *patio* 2 (Table 1). At subsequent fermentation stages, their growth became undetectable or it was so scarce that no statistical tests could be properly carried out.

3.2. Diversity and enumeration of halophilic/alkaliphilic bacteria in green table-olive fermentations

A total of 203 halophilic/alkaliphilic isolates were selected attending to the morphotyping criteria described above. These isolates could be clustered after UPGMA analysis in a phylogenetic dendrogram according to their RAPD-PCR profiles obtained with primer OPL5 (Fig. S2). As a result, up to 65 distinct genotypes (strains) could be distinguished exhibiting similarity indexes ≥ 80% (Fig. S2). For further molecular identification, up to 92 isolates, belonging to 61 different strains, were selected for partial 16S rRNA gene sequencing (Fig. S2). Additionally, in order to improve

Table 1
Averaged halophilic/alkaliphilic bacterial loads in twenty fermenters along the three (initial, middle and final) fermentation stages of Spanish-style green olive fermentations in two fermentation yards (*patios*) obtained in the culture media used in this study (GYECS and RCMAS).

Fermentation yard	Fermenter	Fermentation stage					
		Initial		Middle		Final	
		GYECS	RCMAS	GYECS	RCMAS	GYECS	RCMAS
Patio#1	1	5.73 (0.00)[a]	5.72 (0.01)	ND[b]	ND	ND	ND
	2	5.11 (0.03)	5.11 (0.00)	ND	ND	ND	ND
	3	5.80 (0.01)	5.77 (0.00)	ND	ND	ND	ND
	4	1.88 (0.15)	2.00 (0.00)	ND	ND	ND	ND
	5	6.41 (0.01)	6.52 (0.01)	ND	ND	ND	ND
	6	5.16 (0.03)	5.18 (0.04)	ND	ND	ND	ND
	7	2.18 (0.15)	2.40 (0.00)	ND	ND	ND	ND
	8	5.30 (0.01)	5.28 (0.02)	ND	ND	ND	ND
	9	5.19 (0.03)	5.10 (0.00)	ND	ND	ND	ND
	10	4.44 (0.00)	4.48 (0.00)	2.54 (0.00)	2.70 (0.00)	ND	ND
	Average[c]	4.72 (1.43) [n = 10]	4.76 (1.38) [n = 10]	2.54 (0.00) [n = 1]	2.70 (0.00) [n = 1]	–[d]	–
Patio#2	1	7.31 (0.02)	6.08 (0.01)	2.40 (0.00)	2.18 (0.00)	2.48 (0.00)	2.65 (0.00)
	2	7.31 (0.02)	7.26 (0.01)	2.40 (0.00)	2.54 (0.00)	ND	ND
	3	6.12 (0.01)	5.99 (0.01)	ND	ND	ND	ND
	4	6.41 (0.01)	6.34 (0.01)	ND	2.18 (0.00)	ND	ND
	5	6.14 (0.04)	6.16 (0.01)	1.70 (0.00)	1.70 (0.00)	ND	ND
	6	7.60 (0.01)	7.48 (0.01)	ND	2.18 (0.00)	ND	ND
	7	7.43 (0.00)	7.32 (0.03)	2.40 (0.00)	2.30 (0.00)	2.88 (0.03)	3.06 (0.02)
	8	7.55 (0.01)	7.32 (0.02)	3.15 (0.00)	3.20 (0.01)	ND	1.70 (0.02)
	9	7.26 (0.02)	5.84 (0.00)	2.48 (0.00)	2.65 (0.00)	1.70 (0.00)	2.18 (0.00)
	10	5.65 (0.03)	6.28 (0.03)	2.90 (0.00)	2.98 (0.02)	ND	ND
	Average[c]	6.88 (0.68) [n = 10]	6.61 (0.62) [n = 10]	2.49 (0.42) [n = 7]	2.43 (0.43) [n = 9]	2.35 (0.49) [n = 3]	2.40 (0.51) [n = 4]
	Sig.	*	*	–	–	–	–

[a] Total counts are expressed as the mean values of log CFU/ml based on duplicate analyses made for each sample; standard deviation of the mean (SEM) is shown in parentheses.
[b] ND, not detected.
[c] Averaged halophilic/alkaliphilic bacterial loads, considering only those fermenters (number in square brackets) showing growth of these bacteria.
[d] –not enough data to carry out the statistical test. Sig.: statistical significance considering both *patios* (U Mann–Whitney's test; *for $P < 0.05$).

molecular identification, some strains preliminary identified as *Halolactibacillus* sp. and *Marinilactobacillus* sp. were subjected to (almost) complete sequencing of their 16S rRNA (Fig. S2). Subsequently, the 16S rRNA sequences obtained could be grouped into the 13 phylotypes shown in Table 2, where the bacterial species showing maximum similarity is indicated along with additional species exhibiting ≥ 98.5% similarity. The partial or complete 16S rRNA gene sequence of one representative strain of each phylotype was submitted to the GenBank database (accession numbers in Table 2). Finally, the phylogenetic relationships between 16S rRNA gene partial sequences of these representative strains and those of closest relative species are illustrated in Fig. 1. All of the representative strains could be affiliated to at least 13 distinct species, belonging to 11 different genera.

A summary of the halophilic/alkaliphilic bacterial species isolated in this study as well as the number of isolates and strains

Table 2
Molecular identification of halophilic/alkaliphilic bacterial strains isolated from Spanish-style green table-olive fermentations through 16S rRNA gene sequence homology.

Strain	Length (bp)	Accession number	Closest relative sequence (accession number)	Similarity (%)
Aerococcus sp. G18.53 (2)[a]	423	KT336460	*Aerococcus urinaeequi* IFO 12173 (D87677)[b]	99.7
Alkalibacterium sp. G17.65 (26)	460	KT336461	*Alkalibacterium pelagium* T143-1-1^T (AB294166)[c]	100
Alkalibacterium psychrotolerans G18.55 (1)	427	KT336462	*Alkalibacterium psychrotolerans* IDR2-2^T (AB125938)	99.7
Amphibacillus tropicus J33.61 (15)	477	KT336463	*Amphibacillus tropicus* Z-7792^T (AF418602)	98.5
Catenococcus thiocycli G20.61.2 (3)	463	KT336464	*Catenococcus thiocycli* DSM 9165^T (HE582778)[d]	99.1
Enterococcus olivae G12.61 (4)	464	KT336465	*Enterococcus olivae* IGG16.11^T (JQ283454)	100
Halolactibacillus sp. G13.57 (9)	1453	KT372895	*Halolactibacillus halophilus* M2-2^T (AB196783)[e]	99.0
Halomonas mongoliensis G20.66 (1)	669	KT336467	*Halomonas mongoliensis* Z-7009^T (AY962236)	99.3
Marinilactibacillus sp. G11.53 (9)	460	KT336468	*Marinilactibacillus psychrotolerans* M13-2^T (AB083406)[f]	100
Marinilactibacillus sp. G13.51 (10)	1407	KT336469	*Marinilactibacillus piezotolerans* LT20^T (AY485792)[g]	96.1
Natronobacillus azotifigens G31.52 (6)	477	KT336471	*Natronobacillus azotifigens* 24 KS-1^T (EU143681)	100
Streptohalobacillus salinus G14.54 (2)	424	KT336472	*Streptohalobacillus salinus* H96B60^T (FJ746578)	100
Vibrio sp. J2.62 (4)	464	KT336474	*Vibrio olive* IGJ1.11^T (JQ283456.1)	98.0

[a] In brackets, the number of isolates whose 16S rRNA gene sequence showed a similarity ≥ 98.5% with the 16S rRNA gene sequence submitted to the GenBank database. Further species that are not distinguishable by 16S rRNA gene sequence and/or have a similarity value ≥ 98.5%
[b] *Aerococcus viridans*:
[c] *Alkalibacterium indicireducens*/*thalassium*:
[d] *Vibrio maritimus*/*sagamiensis*:
[e] *Halolactibacillus miurensis*:
[f] *Marinilactibacillus piezotolerans*:
[g] *Marinilactibacillus psychrotolerans*:

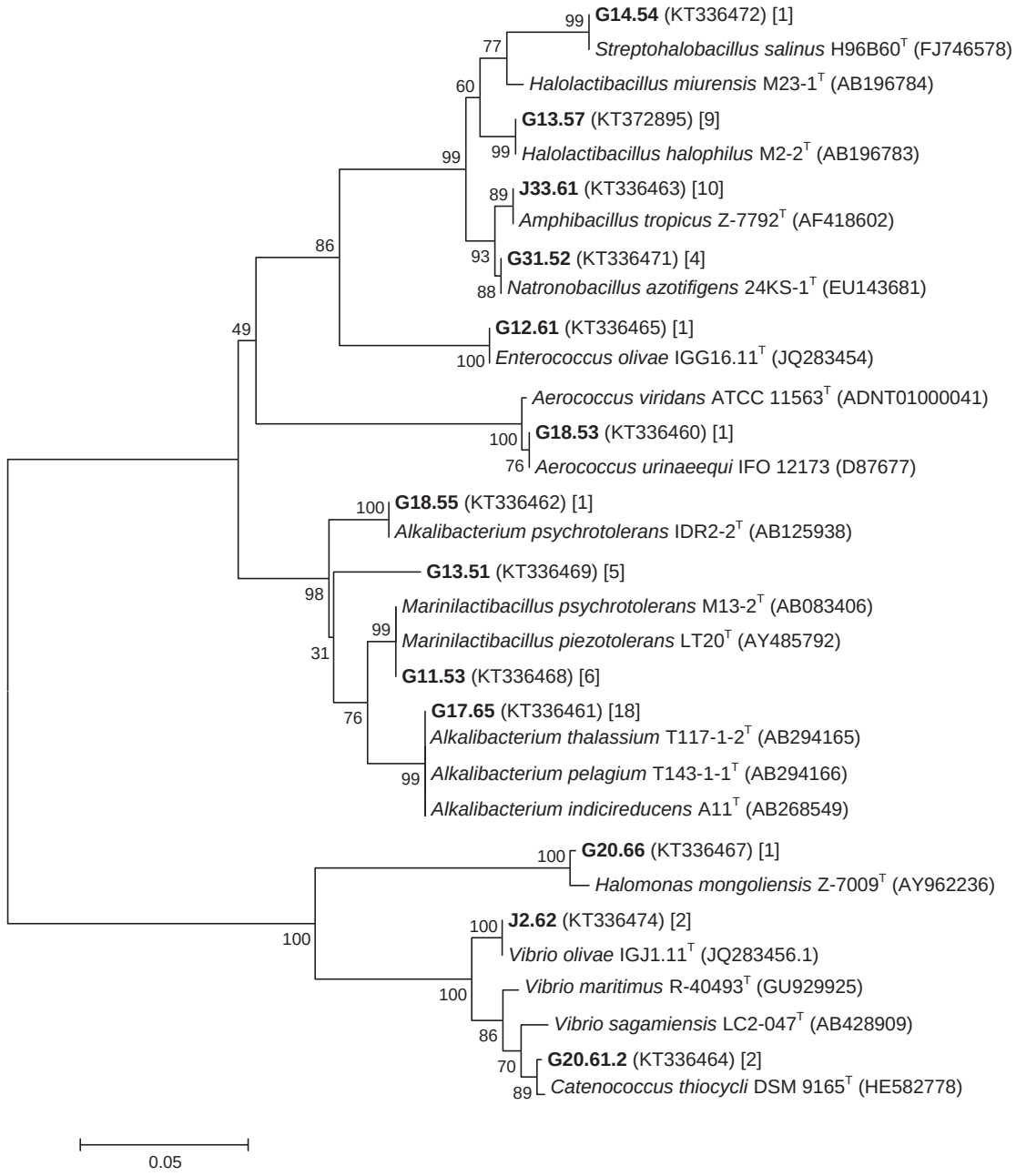

Fig. 1. Phylogenetic relationships based on comparison of partial 16S rRNA gene sequences (427 nucleotide positions) of halophilic/alkaliphilic bacterial strains isolated in this study and the type strains of the most closely related species. Strain names are shown in boldface. GenBank accession numbers are given in parentheses. Bootstrap values (%), calculated from 1000 resamplings using the neighbour-joining method, are shown at the nodes for values ≥ 50%. The number of strains sharing a similar (≥98.5%) partial 16S rRNA gene sequence is shown in square brackets. Bar, 0.05 changes per nucleotide position.

along the three stages of the olive fermentations in the two *patios* studied here is shown in Table 3. Also, the number of fermenters from which a given species could be isolated as well as the count range at which it was present is reported in Table 3. On the other hand, the genotype frequency of these species at the genus level in the 20 fermenters of the two *patios* under study is shown in Fig. 2.

Very similar species composition was recovered using RCMAS or GYECS culture media. However, some species such as *E. olivae*, which had been isolated in a previous study only in *patio* 2 (Lucena-Padrós et al., 2014c), and two species, *Catenococcus thiocycli* and *Halomonas mongoliensis*, plus an unidentified isolate were obtained only in GYECS (Table 3). Furthermore, it was remarkable the prevalence of isolates belonging to the HALAB group, for 35 (64%) and 98 (66%) isolates could be collected from *patio* 1 and 2, respectively. Their presence was ubiquitous in the fermenters under study (Table 3 and Fig. 2), although limited to the initial fermentation stage (Table 3). On the other hand, only two species, shared by both *patios*, i.e. *Amphibacillus tropicus* and *Natronobacillus azotifigens*, could be isolated at the middle and/or final fermentation stages (Table 3).

Fig. 3 shows, through a proportional Venn diagram, the number of microbial genotypes isolated at both *patios* as well as the number of species and genera they belong to. Up to 7 distinct genotypes were shared by both *patios*, belonging to the 6 microbial species

Table 3
Halophilic and alkaliphilic bacterial species isolated along Spanish-style green table-olive fermentations in two different fermentation yards ("*patios*").

Bacterial species	Fermentation stage			Total[a] isolates	Total[b] strains	No.[c] ferm.	Count range[d] (log CFU/ml)
	Initial	Middle	Final				
Patio#1							
Marinilactibacillus psychrotolerans/piezotolerans	28[e]	0	0	28	1	8	1–4
Vibrio olivae[f,g]	13	0	0	13	3	7	1–3
Amphibacillus tropicus	4	1	0	5	3	2	1–4
Alkalibacterium indicireducens/pelagium/thalassium	4	0	0	4	4	3	2–4
Halolactibacillus halophilus/miurensis	2	0	0	2	2	2	1–2
Natronobacillus azotifigens	2	0	0	2	1	1	1
Marinilactibacillus sp.[h]	1	0	0	1	1	1	3
Total isolates[i]	54	1	0	55[j]			
Total strains[k]	15	1	0		15[l]		
Species richness	7	1	0	7[m]			
Patio#2							
Alkalibacterium indicireducens/pelagium/thalassium	32[e]	0	0	32	16	8	3–5
Halolactibacillus halophilus/miurensis	31	0	0	31	8	7	3–5
Marinilactibacillus sp.[h]	22	0	0	22	5	8	1–5
Amphibacillus tropicus	0	13	7	20	10	9	1
Streptohalobacillus salinus	10	0	0	10	1	4	1–5
Marinilactibacillus psychrotolerans/piezotolerans	11	0	0	11	6	3	3–5
Enterococcus olivae[f,n,o]	6	0	0	6	1	4	2–5
Natronobacillus azotifigens	0	6	0	6	4	4	1
Aerococcus viridans/urinaeequi[f]	3	0	0	3	1	3	3–5
Alkalibacterium psychrotolerans	2	0	0	2	1	2	3–4
Catenococcus thiocycli[o]	3	0	0	3	2	3	1
Not identified[o]	1	0	0	1	1	1	4
Halomonas mongoliensis[o]	1	0	0	1	1	1	3
Total isolates[i]	122	19	7	148[j]			
Total strains[k]	43	10	7		57[l]		
Species richness	10	2	1	12[m]			

[a] Total isolates of a specific bacterial species.
[b] Total strains of a specific bacterial species.
[c] Number of fermentors, out of a total of 10, from which a specific bacterial species was isolated in each *patio*.
[d] Colony count range at which that bacterial species was isolated.
[e] Number of isolates of that bacterial species at that fermentation stage.
[f] Bacterial species which have been previously detected and reported in Lucena-Padrós et al., 2014b.
[g] *Vibrio olivae* was previously identified as *Vibrio furnissii/fluvialis* in Lucena-Padrós et al., 2014b and 2014c.
[h] The relatively low (≤97%) 16S rDNA homology of these isolates with other bacterial species in the data banks could indicate that they might be at least a novel species.
[i] Total isolates at each fermentation stage.
[j] Total isolates in each *patio*.
[k] Total strains at each fermentation stage.
[l] Total strains at each *patio*.
[m] Total species richness.
[n] *Enterococcus olivae* was previously identified as *Enterococcus saccharolyticus* in Lucena-Padrós et al., 2014b.
[o] Species which have been isolated only in GYEC media.

and 5 different genera also shown in Fig. 3. For these shared species, the total number of genotypes found for each of them ranged from 4 to 18 (Fig. 3).

Finally, it is important to mention that, to our knowledge and with the exception of just three species, i.e. *E. olivae* (Lucena-Padrós et al., 2014c), *V. olivae* (previously described as *V. furnissii/fluvialis* J1.11v in Lucena-Padrós et al., 2014c) and *A. viridans/urinaeequi* (González-Cancho and Durán-Quintana, 1981; Lucena-Padrós et al., 2014b), the rest of bacterial species, i.e. 10 species, had not been isolated before from any table olive fermentation.

3.3. Biodiversity analyses

Comparisons of richness (R), diversity (H′) and dominance (D) indexes of the overall genotypes between both *patios* are shown in Fig. 4, where H′ and D are calculated both at the species and genus level. Statistical differences were found in all indexes between both *patios*. R and H′ indexes were lower in *patio* 1 than in *patio* 2. In contrast, the highest concentration of dominance was associated to *patio* 1.

On the other hand, when the bacterial species composition of both *patios* was evaluated using different similarity indexes, the estimated values were 0.43 and 0.86 for Jaccard and Morisita-Horn indexes, respectively. When Jaccard index was re-calculated taking into account all of the bacterial species isolated during the first fermentation stage, previously described for these same samples in Lucena-Padrós et al. (2014b) and excluding repeated species, its value was 0.20. However, Morisita-Horn index could not be re-calculated in this manner because of the existence of highly dominant species such as *L. pentosus* and *A. viridans/urinaeequi* (Lucena-Padrós et al., 2014b) which could bias the result.

4. Discussion

This study has corroborated and expanded previous results obtained through a culture-independent technique such as PCR-DGGE applied to samples of fermenting brines obtained from Spanish-style green table-olive fermentations. Thus, the presence

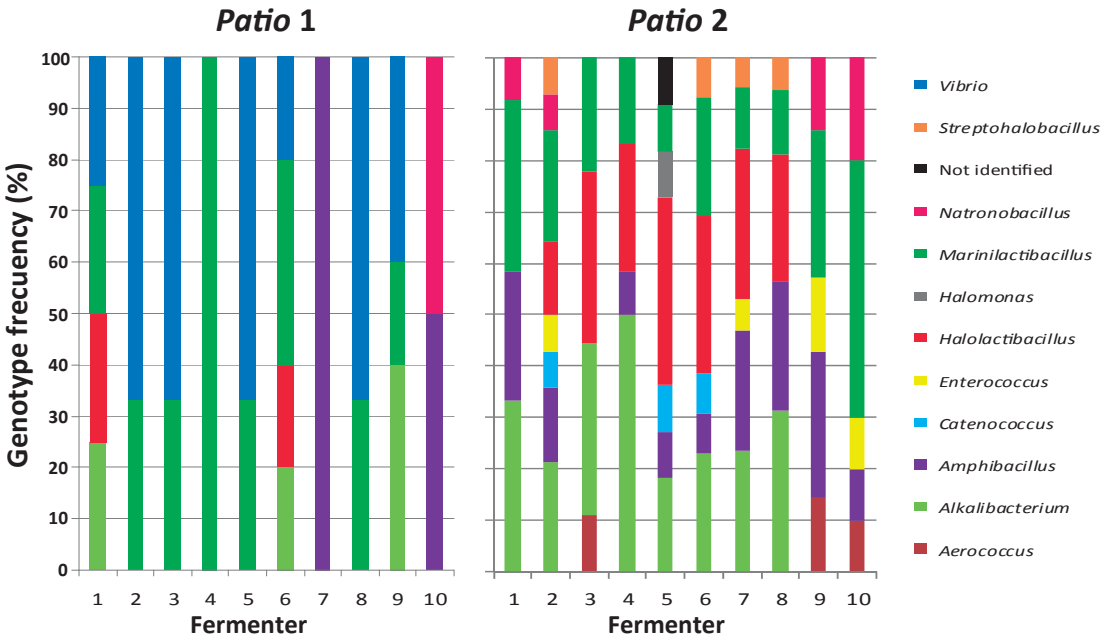

Fig. 2. Genotype frequency of halophilic/alkaliphilic bacterial genera in the overall Spanish-style green table-olive fermentations detected in a total of 20 fermenters located at two different fermentation yards (*patios*).

of halophilic and alkaliphilic bacteria in these samples, predicted by PCR-DGGE (Lucena-Padrós et al., 2015b), has been corroborated after the isolation of up to 203 isolates belonging to at least 13 different species. In the previous, cognate culture-dependent study (Lucena-Padrós et al., 2014b) just three of these species could be isolated, indicating the need of special selective media to assess this many times overlooked part of the characteristic table-olive fermentation microbiota. Although results were very similar with both selective media used here, i.e. RCMAS and GYECS, the fact that some species were only isolated in GYECS suggested that this culture medium could be more appropriated to rescue the halophilic/alkaliphilic microbiota associated to this fermentation.

A statistically significant difference was found in the total counts of halophilic/alkaliphilic bacteria between *patios* 1 and 2 (Table 2). In addition, species richness was higher in *patio* 2 (12 species) than in *patio* 1 (7 species) (Table 3). This result could be due to the fact that in *patio* 1 brines were routinely acidified with HCl as soon as alkali-treated olives were covered in brine (Lucena-Padrós et al., 2014b). This practise, however, is not carried out at that moment of the fermentation in *patio* 2. At this initial stage, averaged pH values were 5.7 and 7.43 in the fermentation brines of *patios* 1 and 2, respectively (Table S1). Therefore, as otherwise it would be anticipated, early acidification appeared to reduce both growth and diversity of halophilic/alkaliphilic bacteria in Spanish-style olive fermentations. On the other hand, NaCl concentration in the brines at equilibrium (first week of fermentation) was 7.76 and 5.88 in the fermenters of *patio* 1 and 2, respectively (Table S1). The less stringent conditions regarding NaCl concentration in *patio* 2 could also contribute to explain the higher counts and halophilic/alkaliphilic species richness observed in this *patio*. As expected, virtually all this

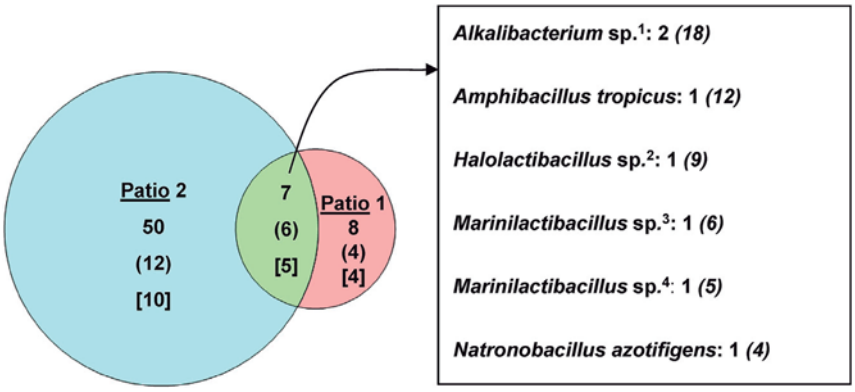

Fig. 3. Number of microbial genotypes, and the species and genus they belong to, shared between the fermenting brines at two fermentation yards (*patios*) during Spanish-style green olive fermentation. The proportional Venn diagram indicates the number of genotypes which have only been isolated at each *patio*, along with the number of species (in brackets) and genera (in square brackets) they belong to. The intersection of this Venn diagram represents the number of genotypes which are shared by both *patios*, as well as the number of species and genera they belong to. The text box indicates the species and the number of genotypes of these species shared by both *patios*. In brackets, the total number of genotypes found for each species. [1]Included *Alkalibacterium indicireducens/pelagium/thalassium*; [2] Included *Halolactibacillus halophilus/miurensis*.;[3]Included *Marinilactibacillus psychrotolerans/piezotolerans*.; [4]Possible novel species, whose closest relative are *Marinilactibacillus psychrotolerans/piezotolerans*.

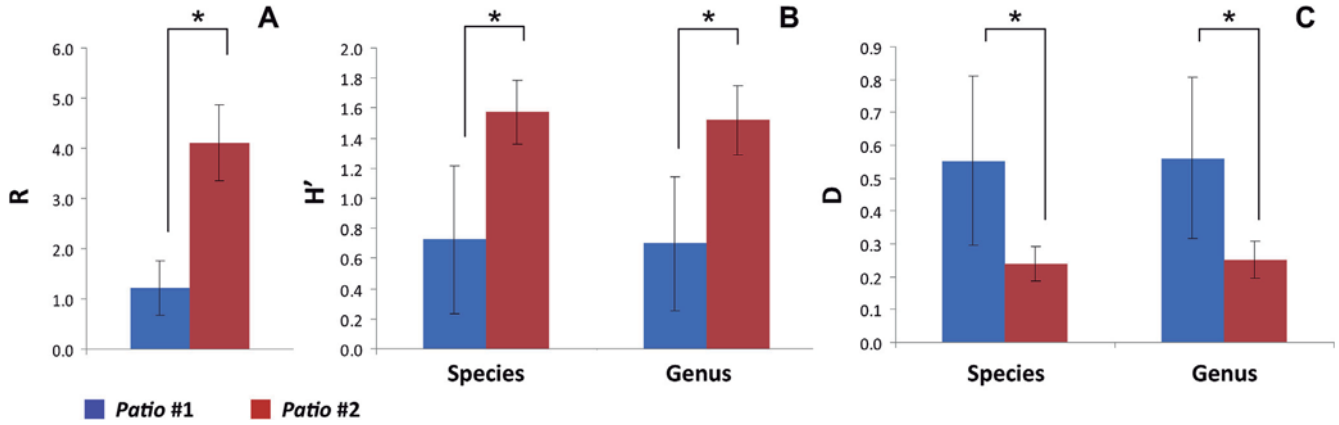

Fig. 4. Richness, diversity and dominance indexes of halophilic/alkaliphilic microbial genotypes found in the fermentation brines at two Spanish-style table-olive fermentation yards (*patios*) (n = 10 at each *patio*). Panel A: Margalef's index of genotype richness (R); Panel B: Shannon–Weaver's index of diversity (H′); Panel C: Simpson's index of dominance (D). H′ and D indexes are calculated at the species as well as the genus levels, as indicated. Data are shown as mean values with SEM. *Statistically significant difference ($p < 0.05$).

microbiota could be isolated only at the initial fermentation stage, i.e. when salt concentration and alkalinic pH are still adequate. In fact, the two only exceptions were the species *A. tropicus* and *N. azotifigens*, which have been described as obligate alkaliphilic and highly salt tolerant (Zhilina et al., 2001; Sorokin et al., 2008). The fact of isolating these two species at fermentation stages when pH values were about 4.3 in both *patios* (Lucena-Padrós et al., 2014b) could be actually due to their ability to form resistant endospores, for they have been described to grow at pH ranges 8.5–11.5 and 7.5–10.6 for *A. tropicus* and *N. azotifigens*, respectively (Zhilina et al., 2001; Sorokin et al., 2008).

It was remarkable the ubiquitous presence of HALAB in both *patios*, whose metabolism, especially the production of lactic acid under alkaline conditions (Ntougias, 2012), undoubtedly contributed to the reduction of the initial highly alkaline pH values of the brines. This in turn should have facilitated the creation of more adequate conditions for the growth of common LAB, such as *L. pentosus*, which can then take over and complete the fermentation. As far as we know, up to 10 bacterial species had not been isolated before from any table olive fermentation, thus demonstrating the value of microbial ecology studies where combined culture-dependent and independent techniques synergistically enhance our knowledge of the real situation in a complex ecosystem such as olive fermentation. In addition, one of the species isolated in both *patios* has been tentatively classified as *Marinilactibacillus* sp. However, the very low homology (96.1%) of the complete 16S rRNA gene of these isolates to other bacterial species suggested that this could constitute at least a novel species. We are currently working out this subject.

Biodiversity at the strain level was assessed through RAPD. In general, strains clustered well into a dendrogram (Fig. S2), showing discrete groups which could well correspond to single species. However, the fact that in some cases it was not possible to distinguish among two or three different species of the same genus using just 16S rRNA gene sequence made it impossible to determine whether this clustering corresponded to actual different species. As expected, the value obtained for the diversity index (H′) was significantly higher in *patio* 2, while dominance was more characteristic of *patio* 1, where a few species such as *Marinilactibacillus psychrotolerans* and *V. olivae* dominated in most of the fermenters. In contrast, up to 4 species appeared to be ubiquitous in the fermenters of *patio* 2 (Table 3). The value obtained for Jaccard index when considering just the halophilic/alkaliphilic microbiota (0.43) was ca. double that obtained when considering the overall bacterial microbiota during the initial fermentation stage in these same fermenters (0.20; Lucena-Padrós et al., 2014b). This could indicate that the halophilic/alkaliphilic microbiota was more similar between both *patios* than the overall microbiota at this stage. Such observation is probably a consequence of the dominance of these species at the first fermentation stage, reflecting a good adaptation to the high salt/high pH conditions which are characteristic of this table olive preparation at this stage. In addition, that indication was reinforced by the detection of up to 7 genotypes which were shared between both *patios*, perhaps indicating that specialised strains are necessary due to the extreme environmental conditions at this stage of the Spanish-style table-olive fermentations. Also, these results could indicate a common origin of these strains and this point is currently under investigation in our laboratory. Finally, the relatively high value (0.86) obtained for Morisita-Horn index, used to quantitatively compare the similarity of species composition, suggested that codominance in both *patios* was carried out by the same species.

This study revealed that the presence of halophilic and alkaliphilic bacteria was widespread among the fermenters of Spanish-style green table olives at the initial fermentation stage. The source of these bacteria is most probably the actual fermentation environment where, selected by the very stringent conditions of pH and salt content at the initial fermentations stages, these halophilic and alkaliphilic bacteria remain season after season in the same *patio*. A suggested origin of these microbiota is the salt supply which, in Spain, is usually of marine origin. The marine origin of many of these species has been indicated by several authors (Ishikawa et al., 2003, 2005, 2009, among others). However, a number of alkaliphilic and/or highly alkali and halo-tolerant bacterial species have been detected or isolated in effluents such as the lyes and washing water employed in the processing steps previous to the actual Spanish-style table olive fermentation (De Castro et al., 2002; Quesada et al., 2007; Ntougias and Russel, 2000, 2001). This fact could suggest that the raw olive fruits could also be a source of some typical halophilic and alkaliphilic species found at the initial fermentation stage of Spanish-style green olives. Some authors have actually associated this microbiota to plant material as it is the case of *Alkalibacterium* species in indigo fermentation liquor (Yumoto et al., 2004, 2008; Nakajima et al., 2005; Aino et al., 2010) or dark fire-cured tobacco leaves (Di Giacomo et al., 2007). Known the relatively high similarity at the species as well the strain levels shown by the fermentation brines at both *patios* studied, it appeared that this table-olive elaboration process and its special

conditions have selected specific species and genotype patterns due to their specific, well adapted metabolism. In this sense, the profuse isolation of HALAB, which are the only known microorganisms able to achieve lactate fermentation under highly alkaline conditions while being quite halotolerant (Ntougias, 2012), was noteworthy. These bacteria can certainly contribute to the conditioning of the fermenting brines so that the microbiota characteristic of the middle fermentation stage, i.e. LAB such as *L. pentosus*, can thrive and accomplish characteristic Spanish-style table-olive fermentations. Finally, considering the results obtained in this study, we suggest the need of routinely introduce specific, selective media to study the evolution of the halophilic/alkaliphilic microbiota during, at least, the initial fermentation stage of Spanish-style green table-olive fermentations. The presence of this bacterial group appears to be a characteristic of this food fermentation at that stage, and its decline can indicate that the middle, or second, fermentation stage has started.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2015.09.006.

References

Abdelkafi, S., Labat, M., Casalot, L., Chamkha, M., Sayadi, S., 2006. Isolation and characterization of *Halomonas* sp. strain IMPC, a p-coumaric acid-metabolising bacterium that decarboxylates other cinnamic acids under hypersaline conditions. FEMS Microbiol. Lett. 255, 108–114.

Abriouel, H., Benomar, N., Lucas, R., Gálvez, A., 2011. Culture-independent study of the diversity of microbial populations in brines during fermentation of naturally-fermented Aloreña green table olives. Int. J. Food Microbiol. 144, 487–496.

Aino, K., Narihiro, T., Minamida, K., Kamagata, Y., Yoshimune, K., Yumoto, I., 2010. Bacterial community characterization and dynamics of indigo fermentation. FEMS Microbiol. Ecol. 74, 174–183.

Altschul, S.F., Madden, T.L., Schaeffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Aponte, M., Blaiotta, G., La Croce, F., Mazzaglia, A., Farina, V., Settanni, L., Moschetti, G., 2012. Use of selected autochthonous lactic acid bacteria for Spanish-style table olive fermentation. Food Microbiol. 30, 8–16.

Cocolin, L., Alessandria, V., Botta, C., Gorra, R., De Filippis, F., Ercolini, D., Rantsiou, K., 2013. NaOH-debittering induces changes in bacterial ecology during table olives fermentation. PLoS One 8 (7), e69074. http://dx.doi.org/10.1371/journal.pone.0069074.

De Castro, A., Montaño, A., Casado, F.J., Sánchez, A.H., Rejano, L., 2002. Utilization of *Enterococcus casseliflavus* and *Lactobacillus pentosus* as starter culture for Spanish-style green olive fermentation. Food Microbiol. 19, 637–644.

Delgado, S., Arroyo, R., Martín, R., Rodríguez, J.M., 2008. PCR-DGGE assessment of the bacterial diversity of breast milk in women with lactational infectious mastitis. BMC Infect. Dis. 8, 51.

Di Giacomo, M., Paolino, M., Silvestro, D., Vigliotta, G., Imperi, F., Visca, P., Alifano, P., Parente, D., 2007. Microbial community structure and dynamics of dark fire-cured tobacco fermentation. Appl. Environ. Microbiol. 73, 825–837.

Garrido Fernández, A., García García, P., Brenes Balbuena, M., 1995. Olive fermentations. In: Rehm, H.-J., Reed, G. (Eds.), Biotechnology: Enzymes, Biomass, Food and Feed. VCH, New York, pp. 593–627.

González-Cancho, F., Durán-Quintana, M.C., 1981. Bacterias cocáceas del ácido láctico en el aderezo de aceitunas verdes. Grasas Aceites 32, 373–379.

IOOC (International Olive Oil Council), 2014. World and European table olive figures. Available Online at. http://www.internationaloliveoil.org/. Accessed May 2014.

Ishikawa, M., Nakajima, K., Yanagi, M., Yamamoto, Y., Yamasato, K., 2003. *Marinilactibacillus psychrotolerans* gen. nov., sp. nov., a halophilic and alkaliphilic marine lactic acid bacterium isolated from marine organisms in temperate and subtropical areas of Japan. Int. J. Syst. Evol. Microbiol. 53, 711–720.

Ishikawa, M., Nakajima, K., Itamiya, Y., Furukawa, S., Yamamoto, Y., Yamasato, K., 2005. *Halolactibacillus halophilus* gen. nov., sp. nov. and *Halolactibacillus miurensis* sp. nov., halophilic and alkaliphilic marine lactic acid bacteria constituting a phylogenetic lineage in *Bacillus* rRNA group 1. Int. J. Syst. Evol. Microbiol. 55, 2427–2439.

Ishikawa, M., Tanasupawat, S., Nakajima, K., Kanamori, H., Ishizaki, S., Kodama, K., Okamoto-Kainuma, A., Koizumi, Y., Yamamoto, Y., Yamasato, K., 2009. *Alkalibacterium thalassium* sp. nov., *Alkalibacterium pelagium* sp. nov., *Alkalibacterium putridalgicola* sp. nov. and *Alkalibacterium kapii* sp. nov., slightly halophilic and alkaliphilic marine lactic acid bacteria isolated from marine organisms and salted foods collected in Japan and Thailand. Int. J. Syst. Evol. Microbiol. 59, 1215–1226.

Kim, O.S., Cho, Y.J., Lee, K., Yoon, S.H., Kim, M., Na, H., Park, S.C., Jeon, Y.S., Lee, J.H., Yi, H., Won, S., Chun, J., 2012. Introducing EzTaxon-e: a prokaryotic 16S rRNA Gene sequence database with phylotypes that represent uncultured species. Int. J. Syst. Evol. Microbiol. 62, 716–721.

Kullen, M.J., Sanozky-Dawes, R.B., Crowell, D.C., Klaenhammer, T.R., 2000. Use of the DNA sequence of variable regions of the 16S rRNA gene for rapid and accurate identification of bacteria in the *Lactobacillus acidophilus* complex. J. Appl. Microbiol. 89, 511–516.

Lane, D.J., 1991. 16/23S rRNA sequencing. In: Stackebrandt, E., Goodfellow, M. (Eds.), Nucleic Acid Techniques in Bacterial Systematics. John Wiley and Sons, Chichester, UK, pp. 115–175.

Lucena-Padrós, H., González, J.M., Caballero-Guerrero, B., Ruiz-Barba, J.L., Maldonado-Barragán, A., 2014a. *Enterococcus olivae* sp. nov., isolated from Spanish-style green-olive fermentations. Int. J. Syst. Evol. Microbiol. 64, 2534–2539.

Lucena-Padrós, H., Caballero-Guerrero, B., Maldonado-Barragán, A., Ruiz-Barba, J.L., 2014b. Microbial diversity and dynamics of Spanish-style green table-olive fermentations in large manufacturing companies through culture-dependent techniques. Food Microbiol. 42, 154–165.

Lucena-Padrós, H., Caballero-Guerrero, B., Maldonado-Barragán, A., Ruiz-Barba, J.L., 2014c. Genetic diversity and dynamics of bacterial and yeast strains associated to Spanish-style green table-olive fermentations in large manufacturing companies. Int. J. Food Microbiol. 190, 72–78.

Lucena-Padrós, H., González, J.M., Caballero-Guerrero, B., Ruiz-Barba, J.L., Maldonado-Barragán, A., 2015a. *Vibrio olivae* sp. nov., isolated from Spanish-style green-olive fermentations. Int. J. Syst. Evol. Microbiol. 65, 1895–1901.

Lucena-Padrós, H., Jiménez, E., Maldonado-Barragán, A., Rodríguez, J.M., Ruiz-Barba, J.L., 2015b. PCR-DGGE assessment of the bacterial diversity in Spanish-style green table-olive fermentations. Int. J. Food Microbiol. 205, 47–53.

Magurran, A.E., 1988. A variety of diversities. In: Magurran, A.E. (Ed.), Ecological Diversity and its Measurement. Princeton University Press, Princeton, N.J, pp. 81–99.

Maldonado-Barragán, A., Caballero-Guerrero, B., Lucena-Padrós, H., Ruiz-Barba, J.L., 2013. Induction of bacteriocin production by coculture is widespread among plantaricin-producing *Lactobacillus plantarum* strains with different regulatory operons. Food Microbiol. 33, 40–47.

Nakajima, K., Hirota, K., Nodasaka, Y., Yumoto, I., 2005. *Alkalibacterium iburiense* sp. nov., an obligate alkaliphile that reduces an indigo dye. Int. J. Syst. Evol. Microbiol. 55, 1525–1530.

Ntougias, S., Russell, N.J., 2000. *Bacillus* sp. WW3-SN6 a novel facultatively alkaliphilic bacterium isolated from the washwaters of edible olive. Extremophiles 4, 201–208.

Ntougias, S., Russell, N.J., 2001. *Alkalibacterium olivoapovliticus* gen. nov., sp. nov., a new obligately alkaliphilic bacterium isolated from edible-olive wash waters. Int. J. Syst. Evol. Microbiol. 51, 1161–1170.

Ntougias, S., 2012. Alkaliphilic lactic acid bacteria: novel sources for genetic engineering and biotechnology. Gene Technol. 1, e102. http://dx.doi.org/10.4172/2329-6682.1000e102.

Quesada, T., Aguilera, M., Morillo, J.A., Ramos-Cormenzana, A., Monteoliva-Sánchez, M., 2007. *Virgibacillus olivae* sp. nov., isolated from waste wash-water from processing of Spanish-style green olives. Int. J. Syst. Evol. Microbiol. 57, 906–910.

Rejano, L., Montaño, A., Casado, F.J., Sánchez, A.H., de Castro, A., 2010. Table olives: varieties and variations. In: Preedy, V.R., Watson, R.R. (Eds.), Olives and Olive Oil in Health and Disease Prevention. Academic Press, Oxford, pp. 5–15.

Ruiz-Barba, J.L., Maldonado, A., Jiménez-Díaz, R., 2005. Small-scale total DNA extraction from bacteria and yeast for PCR applications. Anal. Biochem. 347, 333–335.

Ruiz-Barba, J.L., Jiménez-Díaz, R., 2012. A novel *Lactobacillus pentosus*-paired starter culture for Spanish-style green olive fermentation. Food Microbiol. 30, 253–259.

Saito, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Soll, D.R., 2000. The ins and outs of DNA fingerprinting the infectious fungi. Clin.

Microbiol. Rev. 13, 332–370.
Sorokin, I.D., Zadorina, E.V., Kravchenko, I.K., Boulygina, E.S., Tourova, T.P., Sorokin, D.Y., 2008. *Natronobacillus azotifigens* gen. nov., sp. nov., an anaerobic diazotrophic haloalkaliphile from sodarich habitats. Extremophiles 12, 819–827.
Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739.
Tenover, F.C., Arbeit, R.D., Goering, R.V., Mickelsen, P.A., Murray, B.E., Persing, D.H., Swaminathan, B., 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial typing. J. Clin. Microbiol. 33, 2233–2239.
Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.
Ventorino, V., Chiurazzi, M., Aponte, M., Pepe, O., Moschetti, G., 2007. Genetic diversity of a natural population of *Rhizobium leguminosarum* bv. *viciae* nodulating plants of *Vicia faba* in the vesuvian area. Curr. Microbiol. 55, 512–517.
Yumoto, I., Hirota, K., Nodasaka, Y., Yolota, Y., Hocino, T., Nakajima, K., 2004. *Alkalibacterium psychrotolerans* sp. nov., a psychrotolerant obligate alkaliphile that reduces an indigo dye. Int. J. Syst. Evol. Microbiol. 54, 2379–2383.
Yumoto, I., Hirota, K., Nodasaka, Y., Tokiwa, Y., Nakajima, K., 2008. *Alkalibacterium indicireducens* sp. nov., an obligate alkaliphile that reduces an indigo dye. Int. J. Syst. Evol. Microbiol. 58, 901–905.
Zhilina, T.N., Garnova, E.S., Tourova, T.P., Kostrikina, N.A., Zavarzin, G.A., 2001. *Amphibacillus fermentum* sp. nov. and *Amphibacillus tropicus* sp. nov., new alkaliphilic, facultatively anaerobic, saccharolytic bacilli from Lake Magadi. Microbiology 70, 711–722.

Table S1. Evolution of pH values and NaCl concentrations in brine samples from twenty fermenters of two fermentation yards (*patios*) along the three (initial, middle and final) stages of Spanish-style green olive fermentations.

Ferm. yard	Fermenter	Fermentation stage								
		Initial			Middle			Final		
		time (days)	pH	NaCl (%)	time (days)	pH	NaCl (%)	time (days)	pH	NaCl (%)
Patio #1	1	1	5.90	7.51	35	3.96	6.58	69	3.89	6.35
	2	4	6.20	7.88	38	4.15	6.83	72	4.06	6.37
	3	4	6.10	7.64	38	3.98	7.60	72	3.93	7.60
	4	6	5.00	7.79	40	4.02	7.80	74	3.99	7.80
	5	7	5.90	7.97	41	4.00	7.90	75	3.91	7.80
	6	7	5.85	7.42	41	4.04	7.40	75	3.99	7.40
	7	7	6.11	7.93	41	3.90	7.90	75	3.73	7.65
	8	8	5.92	7.91	42	4.02	7.65	76	3.96	7.90
	9	9	6.03	8.05	43	4.16	8.10	77	4.01	7.95
	10	14	4.00	7.54	48	3.75	7.50	82	3.67	7.55
Patio #2	1	2	7.85	6.31	36	4.40	6.30	73	4.38	6.25
	2	2	8.11	5.88	36	4.45	5.90	73	4.45	5.75
	3	2	7.90	6.17	36	4.44	6.20	73	4.45	6.12
	4	2	8.20	5.59	36	4.53	5.50	73	4.54	5.40
	5	2	7.90	5.59	36	4.52	5.60	73	4.51	5.56
	6	4	6.92	7.00	38	4.00	7.02	75	4.00	7.00
	7	4	7.53	5.40	38	4.50	5.36	75	4.45	5.28
	8	4	7.10	5.30	38	4.02	5.26	75	4.01	5.24
	9	8	6.50	6.20	42	4.03	6.15	79	4.01	6.12
	10	9	6.50	5.40	43	4.12	5.30	80	4.10	5.27

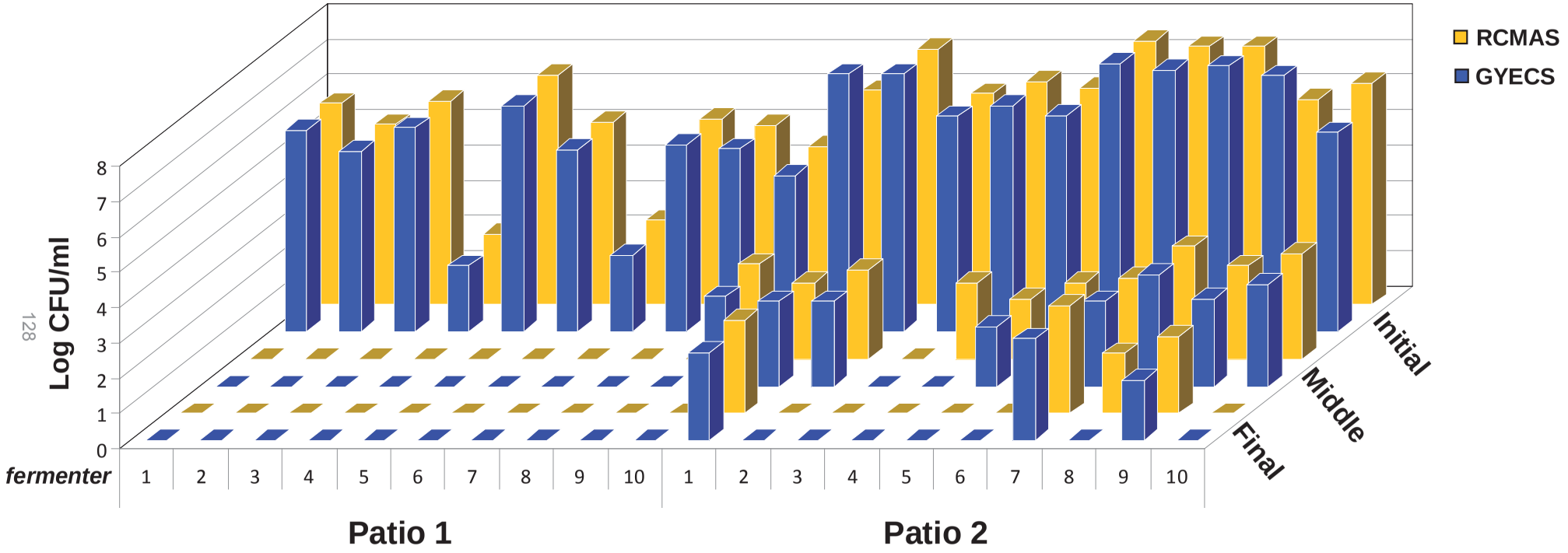
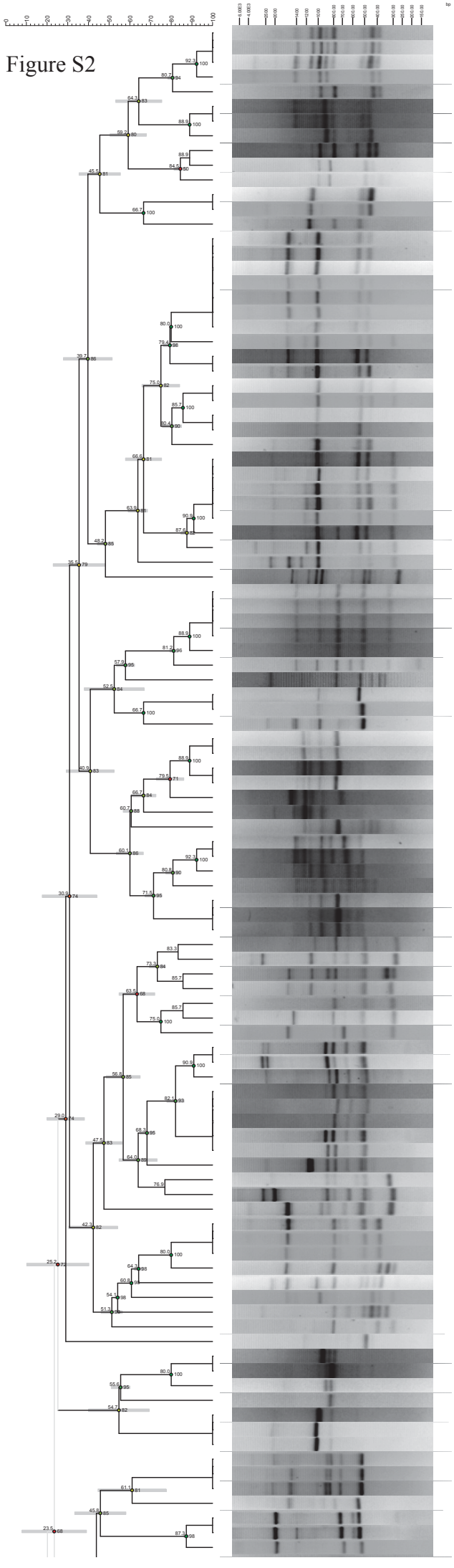
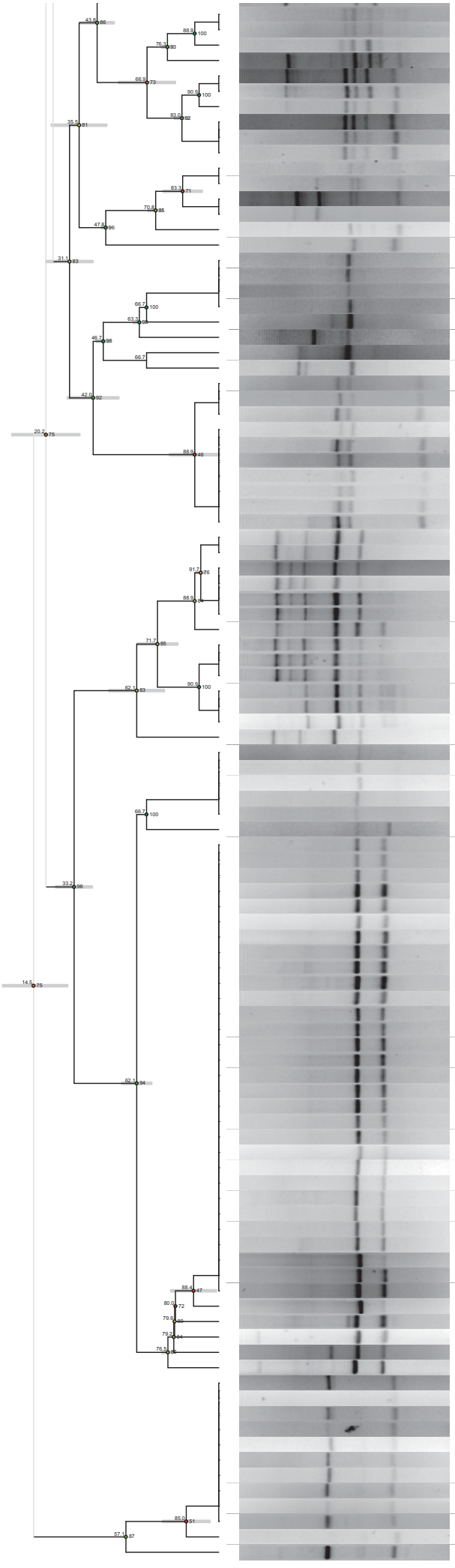


Figure S1. Total counts of halophilic/alkaliphilic bacteria obtained in RCMAS and GYECS culture media along Spanish-style green table-olive fermentations in two different fermentation yards (patios). The analysed fermenter, numbered 1-10 at each patio, is indicated in the X axys. Values are means of log CFU/ml of duplicate samples at each of the three fermentation stages considered in this study, i.e. initial, middle and final. Standard deviations have been omitted for clarity but are shown in Table 2.

Figure S2



Identification	Isolate	Genotype	Patio	Fermenter	Stage
Alkalibacterium indicireducens/pelagium/thalassium	G16.61*	1	2	1	I
Alkalibacterium indicireducens/pelagium/thalassium	G18.62	1	2	3	I
Alkalibacterium indicireducens/pelagium/thalassium	G17.52	1	2	2	I
Alkalibacterium indicireducens/pelagium/thalassium	G19.53	1	2	4	I
Alkalibacterium indicireducens/pelagium/thalassium	G15.62*	1	2	8	I
Amphibacillus tropicus	J13.51*	1	1	10	I
Amphibacillus tropicus	J13.61*	1	1	10	I
Amphibacillus tropicus	**J33.61***	1	1	10	M
Marinilactibacillus sp.	G16.53*	1	2	1	I
Alkalibacterium indicireducens/pelagium/thalassium	G19.61*	2	2	4	I
Alkalibacterium indicireducens/pelagium/thalassium	G19.62	2	2	4	I
Alkalibacterium psychrotolerans	G16.64	1	2	1	I
Alkalibacterium psychrotolerans	**G18.55***	1	2	3	I
Alkalibacterium indicireducens/pelagium/thalassium	G15.64*	3	2	8	I
Marinilactibacillus sp.	G16.63*	2	2	1	I
Marinilactibacillus sp.	G15.53	2	2	8	I
Marinilactibacillus sp.	G17.53	2	2	2	I
Marinilactibacillus sp.	G17.55	2	2	2	I
Marinilactibacillus sp.	G19.54	2	2	4	I
Marinilactibacillus sp.	G20.55	2	2	5	I
Marinilactibacillus sp.	G20.57	2	2	5	I
Marinilactibacillus sp.	G20.52*	2	2	5	I
Marinilactibacillus sp.	G17.54*	3	2	2	I
Marinilactibacillus sp.	G13.63*	3	2	6	I
Marinilactibacillus sp.	G14.51	4	2	7	I
Marinilactibacillus sp.	G18.51*[a]	4	2	3	I
Marinilactibacillus sp.	**G13.51***[a]	4	2	6	I
Marinilactibacillus sp.	G13.53	4	2	6	I
Marinilactibacillus sp.	G16.62	4	2	1	I
Marinilactibacillus sp.	G14.55*[a]	5	2	7	I
Marinilactibacillus sp.	G17.64*	5	2	2	I
Marinilactibacillus sp.	G15.51	5	2	8	I
Marinilactibacillus sp.	G16.51	5	2	1	I
Marinilactibacillus sp.	G19.52	5	2	4	I
Marinilactibacillus sp.	J10.52*[a]	5	1	6	I
Marinilactibacillus sp.	G18.63	5	2	3	I
Halomonas mongoliensis	**G20.66***	1	2	5	I
Not identified	G20.62	1	2	5	I
Enterococcus olivae	G11.63	1	2	10	I
Enterococcus olivae	G11.64	1	2	10	I
Enterococcus olivae	**G12.61***	1	2	9	I
Enterococcus olivae	G14.62*	1	2	7	I
Enterococcus olivae	G17.67*	1	2	2	I
Enterococcus olivae	G12.62*	1	2	9	I
Enterococcus olivae	IGG16.11[Tb]	2	2	1	I
Catenococcus thiocycli	G13.61.2*	1	2	6	I
Catenococcus thiocycli	**G20.61.2***	1	2	5	I
Catenococcus thiocycli	G17.61.2*	2	2	2	I
Amphibacillus tropicus	G56.61	2	2	1	F
Amphibacillus tropicus	G32.51*	2	2	9	M
Amphibacillus tropicus	G37.51*	2	2	2	M
Amphibacillus tropicus	G34.61	2	2	7	M
Amphibacillus tropicus	G54.51*	3	2	7	F
Amphibacillus tropicus	G36.61*	4	2	1	M
Amphibacillus tropicus	G56.51*	5	2	1	F
Amphibacillus tropicus	G35.61	6	2	8	M
Amphibacillus tropicus	G37.61*	6	2	2	M
Amphibacillus tropicus	G52.51*	6	2	9	F
Amphibacillus tropicus	J14.61*	6	1	7	I
Amphibacillus tropicus	G35.62	7	2	8	M
Amphibacillus tropicus	G40.61	7	2	5	M
Amphibacillus tropicus	G54.61	7	2	7	F
Alkalibacterium indicireducens/pelagium/thalassium	G14.64	4	2	7	I
Alkalibacterium indicireducens/pelagium/thalassium	J6.64*	4	1	9	I
Alkalibacterium indicireducens/pelagium/thalassium	G13.52*	5	2	6	I
Alkalibacterium indicireducens/pelagium/thalassium	G13.58*	5	2	6	I
Alkalibacterium indicireducens/pelagium/thalassium	G15.63*	6	2	8	I
Alkalibacterium indicireducens/pelagium/thalassium	G17.62*	6	2	2	I
Alkalibacterium indicireducens/pelagium/thalassium	G19.55*	7	2	4	I
Alkalibacterium indicireducens/pelagium/thalassium	G13.62*	8	2	6	I
Alkalibacterium indicireducens/pelagium/thalassium	G14.53*	8	2	7	I
Alkalibacterium indicireducens/pelagium/thalassium	J15.61	8	1	1	I
Alkalibacterium indicireducens/pelagium/thalassium	G14.52*	8	2	7	I
Alkalibacterium indicireducens/pelagium/thalassium	G15.55	8	2	8	I
Alkalibacterium indicireducens/pelagium/thalassium	G19.63*	8	2	4	I
Alkalibacterium indicireducens/pelagium/thalassium	G13.61	8	2	6	I
Alkalibacterium indicireducens/pelagium/thalassium	G18.61*	8	2	3	I
Alkalibacterium indicireducens/pelagium/thalassium	G13.55*	9	2	6	I
Alkalibacterium indicireducens/pelagium/thalassium	J6.62*	10	1	9	I
Alkalibacterium indicireducens/pelagium/thalassium	G16.52*	11	2	1	I
Alkalibacterium indicireducens/pelagium/thalassium	G16.54*	12	2	1	I
Alkalibacterium indicireducens/pelagium/thalassium	G15.52*	13	2	8	I
Alkalibacterium indicireducens/pelagium/thalassium	G19.51	13	2	4	I
Alkalibacterium indicireducens/pelagium/thalassium	G20.51	13	2	5	I
Alkalibacterium indicireducens/pelagium/thalassium	G14.61*	13	2	7	I
Alkalibacterium indicireducens/pelagium/thalassium	G14.63*	14	2	7	I
Alkalibacterium indicireducens/pelagium/thalassium	G19.56*	15	2	4	I
Alkalibacterium indicireducens/pelagium/thalassium	J10.65*	16	1	6	I
Alkalibacterium indicireducens/pelagium/thalassium	**G17.65***	17	2	2	I
Alkalibacterium indicireducens/pelagium/thalassium	G20.61*	18	2	5	I
Natronobacillus azotifigens	G31.51	1	2	10	M
Natronobacillus azotifigens	G37.62*	1	2	2	M
Natronobacillus azotifigens	**G31.52***	1	2	10	M
Natronobacillus azotifigens	G32.52*	2	2	9	M
Natronobacillus azotifigens	G36.51*	3	2	1	M
Natronobacillus azotifigens	J13.52*	3	1	10	I
Natronobacillus azotifigens	J13.53	3	1	10	I
Marinilactibacillus physichrotolerans/piezotolerans	**G11.53***	1	2	10	I
Marinilactibacillus physichrotolerans/piezotolerans	G12.51*	1	2	9	I
Marinilactibacillus physichrotolerans/piezotolerans	G12.53	1	2	9	I
Marinilactibacillus physichrotolerans/piezotolerans	G11.62*	2	2	10	I
Aerococcus urinaeequi/viridans	G11.65*	1	2	10	I
Aerococcus urinaeequi/viridans	G12.63	1	2	9	I
Aerococcus urinaeequi/viridans	**G18.53***	1	2	3	I



Halolactibacillus halophilus/miurensis	G15.66	1	2	8	I
Halolactibacillus halophilus/miurensis	**G13.57*[a]**	1	2	6	I
Halolactibacillus halophilus/miurensis	G14.58	1	2	7	I
Halolactibacillus halophilus/miurensis	G14.56*[a]	2	2	7	I
Halolactibacillus halophilus/miurensis	G15.54*[a]	3	2	8	I
Halolactibacillus halophilus/miurensis	G14.65	3	2	7	I
Halolactibacillus halophilus/miurensis	G20.63	3	2	5	I
Halolactibacillus halophilus/miurensis	G18.66	3	2	3	I
Halolactibacillus halophilus/miurensis	G15.61	3	2	8	I
Halolactibacillus halophilus/miurensis	G14.57	3	2	7	I
Halolactibacillus halophilus/miurensis	G13.66	4	2	6	I
Halolactibacillus halophilus/miurensis	G14.66	4	2	7	I
Halolactibacillus halophilus/miurensis	G15.68*[a]	4	2	8	I
Halolactibacillus halophilus/miurensis	G20.54	4	2	5	I
Halolactibacillus halophilus/miurensis	G19.64*[a]	5	2	4	I
Halolactibacillus halophilus/miurensis	J10.63*[a]	6	1	6	I
Amphibacillus tropicus	G33.51	8	2	6	M
Amphibacillus tropicus	G35.51*	8	2	8	M
Amphibacillus tropicus	G35.52	8	2	8	M
Amphibacillus tropicus	G39.51	8	2	4	M
Amphibacillus tropicus	G55.51	9	2	8	F
Amphibacillus tropicus	G54.62*	10	2	7	F
Natronobacillus azotifigens	G31.61*	4	2	10	M
Amphibacillus tropicus	J14.51*	11	1	7	I
Streptohalobacillus salinus	G13.56	1	2	6	I
Streptohalobacillus salinus	G17.56	1	2	2	I
Streptohalobacillus salinus	G17.51	1	2	2	I
Streptohalobacillus salinus	G13.64	1	2	6	I
Streptohalobacillus salinus	G13.67	1	2	6	I
Streptohalobacillus salinus	G14.68	1	2	7	I
Streptohalobacillus salinus	G15.65*	1	2	8	I
Streptohalobacillus salinus	G15.61.2	1	2	8	I
Streptohalobacillus salinus	G17.63	1	2	2	I
Streptohalobacillus salinus	**G14.54***	1	2	7	I
Vibrio olivae	J6.55	1	1	9	I
Vibrio olivae	J15.53*	1	1	1	I
Vibrio olivae	IGJ1.11v[Tc]	1	1	2	I
Vibrio olivae	J15.63	1	1	1	I
Vibrio olivae	J1.54	1	1	2	I
Vibrio olivae	J2.52	1	1	8	I
Vibrio olivae	J5.52	1	1	5	I
Vibrio olivae	J6.63*	2	1	9	I
Vibrio olivae	J8.62*	2	1	3	I
Vibrio olivae	J10.64	2	1	6	I
Vibrio olivae	J1.63	2	1	2	I
Vibrio olivae	**J2.62***	2	1	8	I
Vibrio olivae	J5.63	2	1	5	I
Vibrio olivae	J8.53	3	1	3	I
Halolactibacillus halophilus/miurensis	G18.52	7	2	3	I
Halolactibacillus halophilus/miurensis	G17.61*[a]	7	2	2	I
Halolactibacillus halophilus/miurensis	G17.68	7	2	2	I
Halolactibacillus halophilus/miurensis	G19.66	7	2	4	I
Halolactibacillus halophilus/miurensis	G20.64	7	2	5	I
Amphibacillus tropicus	G31.62*	12	2	10	M
Marinilactibacillus physichrotolerans/piezotolerans	J1.61	3	1	2	I
Marinilactibacillus physichrotolerans/piezotolerans	J1.62	3	1	2	I
Marinilactibacillus physichrotolerans/piezotolerans	J2.61	3	1	8	I
Marinilactibacillus physichrotolerans/piezotolerans	J2.63	3	1	8	I
Marinilactibacillus physichrotolerans/piezotolerans	J5.61	3	1	5	I
Marinilactibacillus physichrotolerans/piezotolerans	J5.62	3	1	5	I
Marinilactibacillus physichrotolerans/piezotolerans	J6.61	3	1	9	I
Marinilactibacillus physichrotolerans/piezotolerans	J8.61	3	1	3	I
Marinilactibacillus physichrotolerans/piezotolerans	J10.61*	3	1	6	I
Marinilactibacillus physichrotolerans/piezotolerans	J11.61	3	1	4	I
Marinilactibacillus physichrotolerans/piezotolerans	J15.62*	3	1	1	I
Marinilactibacillus physichrotolerans/piezotolerans	J1.51	3	1	2	I
Marinilactibacillus physichrotolerans/piezotolerans	J1.52	3	1	2	I
Marinilactibacillus physichrotolerans/piezotolerans	J1.53	3	1	2	I
Marinilactibacillus physichrotolerans/piezotolerans	J2.51	3	1	8	I
Marinilactibacillus physichrotolerans/piezotolerans	J5.51	3	1	5	I
Marinilactibacillus physichrotolerans/piezotolerans	J6.51	3	1	9	I
Marinilactibacillus physichrotolerans/piezotolerans	J6.52	3	1	9	I
Marinilactibacillus physichrotolerans/piezotolerans	J6.53	3	1	9	I
Marinilactibacillus physichrotolerans/piezotolerans	J6.54	3	1	9	I
Marinilactibacillus physichrotolerans/piezotolerans	J8.51	3	1	3	I
Marinilactibacillus physichrotolerans/piezotolerans	J8.52	3	1	3	I
Marinilactibacillus physichrotolerans/piezotolerans	J10.51	3	1	6	I
Marinilactibacillus physichrotolerans/piezotolerans	J10.53	3	1	6	I
Marinilactibacillus physichrotolerans/piezotolerans	J11.51	3	1	4	I
Marinilactibacillus physichrotolerans/piezotolerans	J15.51*	3	1	1	I
Marinilactibacillus physichrotolerans/piezotolerans	J15.52	3	1	1	I
Marinilactibacillus physichrotolerans/piezotolerans	G11.54	3	2	10	I
Marinilactibacillus physichrotolerans/piezotolerans	G12.52	3	2	9	I
Marinilactibacillus physichrotolerans/piezotolerans	G12.54	3	2	9	I
Marinilactibacillus physichrotolerans/piezotolerans	G11.52	3	2	10	I
Marinilactibacillus physichrotolerans/piezotolerans	J10.62	3	1	6	I
Marinilactibacillus physichrotolerans/piezotolerans	G11.51*	4	2	10	I
Marinilactibacillus physichrotolerans/piezotolerans	G13.54*	5	2	6	I
Marinilactibacillus physichrotolerans/piezotolerans	G11.61*	6	2	10	I
Halolactibacillus halophilus/miurensis	G18.54*[a]	8	2	3	I
Halolactibacillus halophilus/miurensis	J15.64	8	1	1	I
Halolactibacillus halophilus/miurensis	G14.67	8	2	7	I
Halolactibacillus halophilus/miurensis	G15.67	8	2	8	I
Halolactibacillus halophilus/miurensis	G17.66	8	2	2	I
Halolactibacillus halophilus/miurensis	G18.64	8	2	3	I
Halolactibacillus halophilus/miurensis	G20.65	8	2	5	I
Halolactibacillus halophilus/miurensis	G13.59	8	2	6	I
Halolactibacillus halophilus/miurensis	G13.60	8	2	6	I
Halolactibacillus halophilus/miurensis	G20.53	8	2	5	I
Halolactibacillus halophilus/miurensis	G19.65	8	2	4	I
Halolactibacillus halophilus/miurensis	G13.65*	9	2	6	I

Figure S2

Figure S2. Phylogenetic dendrogram obtained from RAPD-PCR profiles with primer OPL5 of 203 halophilic/alkaliphilic bacterial isolates collected during Spanish-style green table-olive fermentations at two different fermentation yards (patios). The different genotypes (similarity coefficients ≥0.8) found for a given species are indicated, as well as the patio they were isolated from. The actual fermenter, numbered 1-10 at each patio, from which a particular isolate was collected, is indicated in the column labeled "Fermenter". The fermentation stage at which it was isolated is indicated in the column labelled "Stage": I, initial; M, middle; F, final. Scale line at the top indicates the percentage of similarity. The 1 kb Plus DNA ladder (Invitrogen), used to normalize banding patterns, is represented at the top of the figure. In bold, strains whose 16S rRNA sequence has been added to the GenBank database (see accession numbers in Table 1). * Isolates chosen for partial sequencing of the 16S rRNA gene; [a]Isolates whose 16S rRNA gene was (virtually) completely sequenced; [b]Reference strain *Enterococcus olivae* IGG16.11T (Lucena-Padrós *et al.,* 2014a and 2014c); [c]Reference strain *Vibrio olivae* IGJ1.11vT (Lucena-Padrós *et al.,* 2015a; previously described as *Vibrio furnissii/fluvialis* J1.11v in Lucena-Padrós *et al.,* 2014c).

CAPÍTULO 5
DISCUSIÓN GENERAL

Esta Tesis Doctoral ha abordado un tema de investigación que estaba pendiente de ser actualizado, como es el estudio de la microbiota asociada al proceso de elaboración de aceitunas verdes de mesa. En efecto, mientras que el sector del aderezo de aceitunas ha permanecido en constante crecimiento y transformación, modernizándose paulatinamente a lo largo de los últimos años, no ha habido un proceso en paralelo de actualización en los métodos microbiológicos y moleculares para el estudio de la microbiota inherente a esta fermentación de alimentos. Así, es más que previsible que los cambios tecnológicos de las últimas décadas han estado influyendo en la composición y evolución de la microbiota asociada.

Para este estudio se han elegido dos empresas de gran tamaño dedicadas a la elaboración de aceitunas de mesa (4.000 - 8.000 t de producción anual), JOLCA S.A. y GOYA España S.A. (denominadas "patio 1" y "patio 2", respectivamente, en los artículos publicados). La mayor parte de la producción de estas empresas se dedica, precisamente, a la elaboración en verde de aceitunas de mesa, el denominado estilo Español o sevillano. Los patios de producción de ambas empresas están localizados en la provincia de Sevilla, la región geográfica de mayor producción mundial de este tipo de aceitunas de mesa. Asimismo, este estudio se ha realizado sobre aceitunas de la variedad Manzanilla Sevillana, una de las más comercializadas y apreciadas para este tipo de elaboración. En los patios mencionados se analizaron las salmueras de fermentación de un total de 20 fermentadores de 10 t cada uno, tomándose muestras coincidiendo con cada una de las tres fases descritas para esta fermentación. Estas fermentaciones se desarrollaron de forma espontánea (sin la adición de inóculos comerciales) y evolucionaron en la forma habitual desde el punto de vista físico-químico (Lucena-Padrós *et al.,* 2014b; Lucena-Padrós y Ruiz-Barba, 2016).

Otro de los objetivos de esta Tesis Doctoral era garantizar la conservación de las características propias del producto mediante la conservación de la microbiota autóctona inherente al proceso fermentativo. Por este motivo, para estos estudios se han empleado técnicas dependientes de cultivo, combinándolas con técnicas moleculares para lograr un mayor grado de resolución en el proceso de identificación de los aislados obtenidos. Para conseguir tener un enfoque ecológico lo más realista posible, se procuró recuperar la mayor diversidad genética y riqueza de especies presentes, por lo que se aislaron representantes de todos los morfotipos de colonia distintos observables en la batería de medios de cultivo utilizados.

La caracterización a nivel de cepa de la colección de aislados obtenida se hizo mediante la técnica de genotipado RAPD-PCR. Esta técnica ha sido ampliamente utilizada con éxito para la caracterización genética de bacterias lácticas (Rodas *et al.,* 2005) y en la monitorización y/o identificación de inóculos en fermentaciones dirigidas, mediante la inclusión de cepas de referencia en la construcción de los dendrogramas de similitud (Giraffa y Rossetti, 2004; Rossetti y Giraffa 2005; Seseña *et al.,* 2005). De hecho, esta técnica de genotipado es la utilizada con mayor frecuencia para bacterias aisladas de aceitunas de mesa (Botta y Cocolin, 2012), aunque también ha demostrado ser eficaz para el genotipado de levaduras procedentes de estas fermentaciones (Tofalo *et al.,* 2013). A este respecto, hay que tener en cuenta la misma definición de especie microbiana, según la cual las distintas cepas que la constituyen se agrupan en base a su parecido genómico general y a que comparten fenotipos considerados ecológicamente importantes (Vos, 2011). Sin embargo, no todas las cepas pertenecientes a una misma especie microbiana presentan las mismas características fenotípicas (Tailliez *et al.,* 1996). Por tanto, la caracterización genotípica al nivel de cepa contemplada en esta Tesis Doctoral constituye un paso previo y necesario para evaluar el potencial biotecnológico de la colección de microorganismos obtenida. Estos datos serán muy relevantes de cara a diseñar, por ejemplo, un cultivo iniciador (Leroy y De Vuyst, 2004) o deducir el potencial funcional de las distintas cepas y especies microbianas en esta matriz alimentaria (Ravyts *et al.,* 2012). Esto se debe a que, más allá del carácter aparentemente cosmopolita de muchos microorganismos (derivado de una gran plasticidad genómica, que les permite habitar multitud de ambientes distintos, sobrevivir a condiciones físico-químicas extremas o establecer interacciones biológicas diversas), son las características únicas de cada ecosistema las que seleccionan qué poblaciones de microorganismos con actividades metabólicas específicas conforman esas comunidades microbianas concretas, así como la biogeografía o distribución espacial de las mismas y las interacciones que entre ellas se suceden dentro de un ecosistema particular (Konopka, 2006; Prosser *et al.,* 2007; Young *et al.,* 2008).

En el desarrollo de este proyecto de Tesis Doctoral se ha evaluado el principal concepto ecológico para medir la calidad de un hábitat, esto es, la biodiversidad. En este caso, la biodiversidad asociada a la fermentación de aceitunas verdes de mesa en las propias industrias de aderezo. Para ello, se han considerado tanto la variabilidad intraespecífica (diversidad genética) como la variabilidad de una comunidad (riqueza, abundancia y distribución de las especies) (Whittaker, 1977; Bisby *et al.,* 1995; Jost, 2007) mediante el cálculo de diversos

índices de diversidad alfa y beta para establecer comparaciones entre los patios de fermentación estudiados y entre las distintas fases de la fermentación.

5.1 La microbiota de la fermentación de aceitunas verdes de mesa

5.1.1 La fase inicial de la fermentación de aceitunas verdes

Esta es la fase más heterogénea en cuanto a diversidad de especies microbianas. Si bien durante esta etapa se apreció ya el crecimiento, e incluso dominancia, de cepas de la especie *L. pentosus* en uno de los patios estudiados, se observó una situación de codominancia junto con la especie *Aerococcus viridans/urinaeequi*, una especie que, sin embargo, desaparece en los estadios más avanzados de la fermentación. De hecho, por su capacidad de crecer a valores de pH hasta 9,6 y concentraciones de NaCl de hasta el 10%, en el pasado se ha considerado a éste el primer género entre el grupo de bacterias lácticas capaz de crecer en las salmueras de fermentación en aceitunas verdes al estilo Español (González Cancho y Durán Quintana, 1981). Sin embargo, su desarrollo suele ser limitado, dado que esta especie empieza a desaparecer cuando los valores de pH son inferiores a 5,5, siendo indetectable cuando la acidez libre es superior a 0,20% (expresado como g ácido láctico/L). Hay que subrayar que la abundancia de cocos lácticos, junto con un menor crecimiento o incluso desaparición de bacilos Gram-negativos, se ha asociado en el pasado a la producción en fermentadores de gran tamaño (≥10 t de frutos) y a la mejora en las condiciones de higiene (González Cancho y Durán Quintana, 1981). Estas condiciones tienen que ver, principalmente, con una mayor calidad del agua y las salmueras utilizadas, así como con un menor riesgo de contaminación debido a la mayor facilidad de la limpieza de estos fermentadores frente a los tradicionales bocoyes de plástico o madera (González Cancho y Durán Quintana, 1981).

En esta primera fase de la fermentación es en la que se han podido aislar especies bacterianas filogenéticamente más distantes, así como una mayor diversidad de géneros microbianos. Es precisamente el género el que es considerado el rango taxonómico más alto con significado biológico en microbiología (Cowan, 1968), indicándonos que es en esta primera fase cuando se puede apreciar una mayor heterogeneidad metabólica y funcional asociada a este nicho ecológico. En cambio, en los estadios más avanzados predominan las bacterias lácticas de diversas especies de *Lactobacillus* y *Pediococcus*. Por otra parte, dado que la fermentación tiene lugar de forma espontánea y el material de partida no puede ser

esterilizado, es natural que sea también en la fase inicial en la que se detecte una mayor carga de microbiota contaminante. Esto queda posiblemente reflejado por la alta tasa de especies "únicas" (en inglés, *"singlentons"*) que aparecen esporádicamente y que no suelen alcanzar altos rangos de conteos.

Los estudios para la identificación y monitorización específica de poblaciones microbianas extremófilas, tales como bacterias halófilas y/o alcalófilas, realizados mediante técnicas de cultivo, han puesto de manifiesto la importancia de las mismas como microorganismos pioneros en la colonización de este nicho ecológico. Con anterioridad, algunos autores habían señalado la presencia de microorganismos extremófilos tales como bacterias halófilas y/o alcalófilas en efluentes derivados de la industria e incluso sugerido la participación de las mismas en la fermentación (Ntougias y Russel, 2000, 2001; de Castro *et al.,* 2002; Abdelkafi *et al.,* 2006; Quesada *et al.,* 2007; Abriouel *et al.,* 2011; Cocolin *et al.,* 2013a). No obstante, estos estudios revelan por primera vez a esta microbiota como característica de este tipo de elaboración, especialmente durante la primera fase de la fermentación. Así, y pese a la amplia diversidad de especies asociadas a esta primera fase, el mayor parecido en cuanto a composición microbiana entre los patios estudiados se debe principalmente a bacterias halófilas y alcalófilas en base al número de especies compartidas por ambos. Entre estas especies, fueron las bacterias halófilas y alcalófilas del ácido láctico (HALAB) pertenecientes a los tres géneros descritos, *Alkalibacterium, Marinilactibacillus y Halolactibacillus* (Ntougias*,* 2012), las más destacables por su abundancia, diversidad y ubicuidad. Probablemente, la importancia ecológica de las HALABs en el proceso fermentativo se deba en parte a la capacidad de éstas de producir ácido láctico en condiciones alcalinas (Ntougias*,* 2012). Esta capacidad contribuiría decisivamente al descenso inicial de los valores del pH de las salmueras, entorno a pH 10, hasta valores que permitirán el posterior desarrollo de bacterias lácticas comunes, incluyendo la dominancia de la especie alcali-tolerante *L. pentosus* que tendrá lugar, definitivamente, en la fase media de la fermentación de aceitunas verdes. No obstante, el papel que desempeñan estas bacterias dentro esta fermentación parece limitarse principalmente a la fase inicial, en donde se encuentran en mayor cantidad y diversidad, en consonancia con las condiciones físico-químicas de las salmueras de fermentación. Una excepción fue la persistencia de las especies *Amphibacillus tropicus* y *Natronobacillus azotifigens* en las salmueras, las cuales fueron aisladas en ambos patios y en estadios avanzados de la fermentación. Probablemente, la habilidad de sobrevivir de estos alcalófilos obligados en un entorno ácido ($pH \leq 4$) esté vinculada con la capacidad de

estas especies de formar endosporas resistentes (Zhilina *et al.,* 2001; Sorokin *et al.,* 2008). Así, existe la duda razonable de que estas especies estén realmente activas, metabólicamente, durante las siguientes fases de la fermentación.

Por otra parte, hay que tener en cuenta que diferentes prácticas por parte de las empresas durante las operaciones previas a la fermentación, tales como la adición de sal al tratamiento alcalino o la acidificación inicial de las salmueras, ambas realizadas exclusivamente en el patio 1, podrían explicar no sólo las diferencias observables en las características físico-químicas de las salmueras de fermentación (mayor concentración de sal en el equilibrio, niveles de pH inicial inferiores y de acidez libre final mayores en el patio 1 respecto al patio 2) sino también en la composición de las poblaciones microbianas que son detectadas durante la fermentación. La acidificación inicial de las salmueras, obtenida generalmente con adiciones de ácido clorhídrico o de ácido acético, parece reducir la concentración y la diversidad de poblaciones microbianas halófilas y alcalófilas, lo que explicaría las diferencias observadas entre ambos patios a este respecto. También, una posible explicación de porqué en el patio 2 se aprecia una codominancia entre *L. pentosus* y *A. viridans/urenaeequi* durante esta primera fase de la fermentación, o la diferencia entre las especies de *Enterococcus* asociadas a cada patio, se deba a los valores de pH iniciales, más ácidos en las salmueras del patio 1. Estos valores de pH estarían así más próximos a las condiciones óptimas para el crecimiento de los lactobacilos, favoreciendo su rápida implantación en el medio. No obstante, aun cuando algunos autores han señalado la ventaja de la acidificación de las salmueras para acelerar el proceso fermentativo y evitar riesgos de contaminaciones (Borbolla y Alcalá *et al.,* 1969), este tratamiento requiere un especial cuidado si se quieren evitar otras alteraciones, como son las fermentaciones detenidas (González Cancho *et al.,* 1983). Esta alteración se ha observado especialmente en aceitunas Manzanilla, en las que la adición brusca de un ácido fuerte, como es el ácido clorhídrico, para reducir el valor de pH y la alta lejía residual, puede ocasionar la precipitación de proteínas y aminoácidos, dando lugar a una menor disponibilidad de nutrientes en el medio. Este fenómeno puede llegar a provocar la inhibición del crecimiento de bacterias lácticas comunes, lo que tiene como consecuencia la proliferación de levaduras en las salmueras y su implantación como flora dominante (Durán-Quintana *et al.,* 1997).

5.1.2 Las fases media y final de la fermentación de aceitunas verdes

La fase media de la fermentación de aceitunas verdes se caracteriza, microbiológicamente, por el desplazamiento definitivo de la microbiota observada durante la fase inicial a consecuencia del crecimiento exponencial de diversas especies de BAL y al consiguiente descenso de los valores de pH de las salmueras. La especie dominante en esta fase es claramente *L. pentosus,* a la que pueden acompañar otras BAL como *Lactobacillus paracollinoides/collinoides, Pediococcus parvulus, Lactobacillus paracasei, Lactobacillus parafarraginis* o *Lactobacillus coryniformis*, entre otras. Con anterioridad, otros autores encontraron algunas de estas especies durante esta fase de la fermentación (Aponte *et al.,* 2012; Doulgeraki *et al.,* 2013; Montaño *et al.,* 2013). La frecuencia de aparición de estas BAL "acompañantes" puede variar dependiendo de múltiples factores, tales como la localización geográfica o las prácticas propias de cada empresa durante el proceso de elaboración.

La salmuera de fermentación, en esta fase media, resulta ya un caldo de cultivo rico, como refleja el predominio de grupos microbianos con requerimientos nutritivos complejos, como es el caso de cepas de *Lactobacillus* y *Pediococcus*. Estas bacterias lácticas tienen una capacidad de acidificación mayor que las especies presentes durante la primera fase (Battcock y Azam-Ali, 1998; Liu, 2002) y son las principales responsables del consumo de los azúcares procedentes de la pulpa de los frutos y la consiguiente formación de ácidos orgánicos, principalmente ácido láctico. La producción de estos ácidos orgánicos promueve la disminución de los valores de pH de la salmuera hasta niveles en el rango 4,0-4,5, aunque este descenso no es lineal, debido a la salida de sales orgánicas del interior de los frutos que tamponan este descenso (acidez combinada). Estas sales se forman con los restos de hidróxido de sodio utilizado en el tratamiento alcalino típico de esta elaboración ("lejía residual").

Microbiológicamente, la fase final de la fermentación de aceitunas verdes es muy similar a la fase media en cuanto a composición de especies, aunque el número de microorganismos desciende a medida que se agota la materia fermentable. La persistencia de *L. pentosus* y de otras BAL acompañantes tales como *L. paracollinoides/collinoides, P. parvulus, Pediococcus ethanolidurans* o *L. parafarraginis* se hace patente, aunque con concentraciones celulares menores que en la fase anterior. Los valores de pH alcanzan sus mínimos al final de esta fase, normalmente en el rango 3,8-4,2, así como los máximos valores de acidez libre, considerándose apropiados aquellos en el rango 0,7-1,0 g/L. Los valores alcanzados para estos parámetros físico-químicos, junto con una concentración de NaCl por encima del 5%,

garantizan la conservación de los frutos fermentados hasta el envasado final. Sin embargo, en esta fase final pueden aparecer también especies bacterianas, como algunas del género *Propionibacterium*, que pueden empezar a consumir los ácidos orgánicos producidos durante la fermentación, haciendo que los valores de pH suban y comprometiendo al producto ya elaborado en lo que algunos autores han denominado "cuarta fase" de la fermentación de aceitunas verdes. Algunas levaduras de metabolismo oxidativo pueden contribuir también a la aparición de esta anomalía que, sin embargo, suele estar bien controlada en los patios competentes mediante correcciones de sal y pH al final de la fermentación y, especialmente, ante el aumento de la temperatura ambiente con la llegada de la primavera.

5.1.3 Las levaduras durante la fermentación de aceitunas verdes

Los estudios sobre la evolución de las levaduras durante la fermentación de aceitunas verdes de mesa indicaron que éstas presentaron una menor complejidad, al nivel de especie, durante todo el proceso fermentativo respecto a la manifestada por las bacterias. Asimismo, los recuentos de viables fueron siempre inferiores a los alcanzados por las bacterias, así como se observaron una menor riqueza y diversidad de especies. En este sentido, estos resultados son similares a los obtenidos por Bautista-Gallego *et al.,* (2011), tanto por el número de especies encontradas como por las concentraciones totales alcanzadas por las levaduras, esto es, en torno a $6{,}0\times10^3$ ufc/ml. Estas concentraciones, sin embargo, son menores al rango anteriormente descrito para la población de levaduras, que sitúan su concentración entre 10^4 y 10^6 ufc/ml en fermentaciones industriales de aceitunas verdes (Garrido-Fernández *et al*., 1997).

Quizás, el resultado más reseñable de este estudio respecto a la evolución de las levaduras fue el constatar una verdadera sucesión ecológica entre las especies participantes en cada etapa de la fermentación, más allá de una mera coexistencia entre las mismas. En este sentido, lo más destacable fue la sucesión en la especie dominante, es decir, desde cepas de la especie *Saccharomyces cerevisiae* a cepas de la especie *Candida thaimueangensis,* como patrón común en ambos patios.

Aunque la presencia de levaduras *a priori* no parece estrictamente imprescindible para la elaboración de este producto, algunas de las especies que normalmente acompañan a las bacterias lácticas durante la fermentación sí podrían ser beneficiosas (Durán-Quintana *et al.,* 1997; Arroyo-López *et al.,* 2012a) e incluso influir positivamente en las propiedades

organolépticas del producto final (Arroyo-López *et al.,* 2008). Sin embargo, hay que mencionar, como otros autores han manifestado anteriormente, que la importancia ecológica y el papel que desempeñan las levaduras durante este proceso se halla hasta la fecha parcialmente enmascarado debido a la dificultad técnica para establecer una relación entre las distintas especies de levaduras presentes y su actividad metabólica en las salmueras, o incluso las interacciones que establecen con otros microrganismos (Arroyo-López *et al.,* 2012b). Por otra parte, como se ha citado más arriba, ciertas especies de levaduras de metabolismo oxidativo pueden consumir parte de los ácidos orgánicos producidos durante la fermentación (Ruiz Cruz y González Cancho, 1969). Ello haría elevar los valores de pH de las salmueras, permitiendo el crecimiento de ciertos microorganismos alterantes, como ciertas especies del género *Clostridium*, contribuyendo así al establecimiento de la ya citada, y no deseada, cuarta fase de la fermentación de aceitunas verdes.

5.2 La diversidad microbiana durante la fermentación de aceitunas verdes

Los índices de diversidad beta, aquellos que comparan hábitats dentro de un mismo ecosistema, cuando fueron aplicados a la fermentación de aceitunas verdes, mostraron que el cambio en la composición microbiana tiene lugar de forma progresiva conforme avanza la fermentación. Así, la microbiota de ambos patios se torna cada vez más similar a medida que avanza la fermentación, relegando las diferencias a la microbiota accesoria. Todo ello queda también reflejado en la evolución temporal de los principales índices de diversidad alfa, en los que se puede apreciar un aumento progresivo de la dominancia en yuxtaposición con la perdida de diversidad de especies. La madurez de este ecosistema se alcanza durante la fase media de la fermentación, en la que se hace patente la comunidad microbiana clímax para este proceso. Esta comunidad estaría compuesta por las especies mejor adaptadas a su entorno, fuertemente interrelacionadas entre sí y capaces de desplazar al resto de especies oportunistas. Al término de la fermentación, cuando se alcanzan los niveles máximos de acidez libre y se agotan los principales recursos fermentables, se produce un descenso significativo en los niveles de biomasa alcanzados por la comunidad microbiana madura. Esto podría, si no se toman las medidas tecnológicas oportunas (aumento de la concentración de sal, acidificación, etc.) dar lugar al desarrollo de especies microbianas con potencial de producir alteraciones del producto final.

El estudio de la evolución de la diversidad genética a lo largo de la fermentación mostró que, además del recambio de especies microbianas descrito, existe un recambio a nivel de

cepas. Así, se encontraron escasos candidatos capaces de resistir durante todo el proceso. Sin embargo, uno de estos candidatos resultó particularmente llamativo, dado que su perfil genético, obtenido mediante RAPD-PCR, se agrupaba con la cepa bacteriocinogénica *L. pentosus* LPCO10, perteneciente a un inóculo comercial, con porcentajes de similitud ≥80%. Ambas empresas declararon haber usado este inóculo en campañas anteriores, más concretamente en 2007–2008 y 2008–2009 en los patios 1 y 2, respectivamente. Este hecho sugiriere que esta cepa podría ya formar parte de la microbiota endógena de ambos patios, en los que se halló ampliamente distribuida entre los distintos fermentadores analizados. En este sentido, se ha señalado la producción de bacteriocinas como una ventaja en cepas del grupo *L. plantarum* para persistir en las salmueras de fermentación de forma dominante (Ruiz-Barba *et al.,* 1994, 2010; Ruiz-Barba y Jiménez-Díaz, 2012). Adicionalmente, algunos autores han demostrado la capacidad de estas cepas de adherirse a la superficie de distintos materiales, incluyendo la fibra de vidrio de la que están hechos los fermentadores industriales, y formar biofilms (Domínguez-Manzano *et al.,* 2012; Grounta *et al.*, 2015). Por otro lado, también se detectó un genotipo, identificado como una especie del género *Stafilococcus* del grupo epidermidis, presente en ambos patios y capaz de persistir durante todo el proceso fermentativo. Este dato sugiere que habría que mejorar las condiciones de higiene en los patios de fermentación si se quiere evitar que cepas de estas especies, consideradas patógenos oportunistas (Ghebremedhin *et al.,* 2008), acaben implantándose en los mismos.

Resultaría interesante evaluar mejor la dispersión de las cepas "cosmopolitas" detectadas en este estudio, es decir, un total de 68 cepas que fueron aisladas en ambos patios de fermentación. La mayoría de estas cepas (51) pertenecían a la especie dominante, *L. pentosus*, aunque también se detectaron cepas con esta característica en *Alkalibacterium indicidurens/pelagium/thalassium*, *A. tropicus*, *Halolactibacillus halophilus/miurensis, L. paracollinoides/collinoides, Lactobacillus rapi, Marinilactibacillus* sp., *Marinilactibacillus physichrotolerans/piezotolerans*, *N. azotifigens*, *P. ethanolidurans* y *Staphilococcus* sp., entre las bacterias. También entre las levaduras se aislaron cepas cosmopolitas en las especies *C. thaimueangensis*, *S. cerevisiae* y *Hanseniaspora* sp. Un estudio en mayor profundidad de este aspecto podría aclarar si estas cepas dotan al producto de alguna cualidad diferencial, dado su carácter aparentemente autóctono. Estas cepas, bien adaptadas a la elaboración tradicional de este producto dentro de la zona geográfica de mayor producción del mismo, usadas como inóculos podrían aportar un valor añadido al producto elaborado. Por otra parte, la presencia

de las mismas aportaría una cierta "garantía de origen", que incluso podría hacerse constar en las especificaciones del producto final. En esta línea, dentro de cada patio de fermentación también se estudió la dispersión e implantación de las cepas entre los distintos fermentadores analizados. Se podría evaluar así la posible impronta de las distintas cepas en el producto final. Entre estas cepas, cabe destacar en particular las pertenecientes a la especie dominante *L. pentosus*, habiéndose encontrado hasta 28 genotipos ampliamente distribuidos por un número elevado de fermentadores dentro de cada patio de fermentación.

El estudio de los estimadores de diversidad genética reveló una alta riqueza genotípica a lo largo de la fermentación, al igual que lo señalado más arriba para la diversidad de especies. Como en el caso anterior, este hecho podría estar en consonancia con la forma de muestreo elegida, es decir, el aislamiento de todos los morfotipos distintos de colonia en la batería de medios de cultivo utilizados. Los estudios de diversidad y dominancia genéticas se realizaron de forma global, es decir, teniendo en consideración toda la diversidad genética encontrada e incluyendo, por tanto, las bacterias y las levaduras conjuntamente, así como las especies "raras" o "únicas" (*"singletons"*). De esta forma, se observa que el tiempo es un factor influyente (estadísticamente significativo) en ambos patios, si bien no se apreciaron diferencias entre los patios, los cuales presentaron tendencias similares. Esta aproximación resulta, en nuestra opinión, más realista y completa, dado que contempla todas las posibles interacciones biológicas. Esto incluye las que tienen lugar entre las bacterias y las levaduras, como han demostrado algunos autores con anterioridad (Nychas *et al.,* 2002; Arroyo-López *et al.,* 2012b; Domínguez-Manzano *et al.,* 2012; Grounta *et al.*, 2015; León-Romero *et al.*, 2016).

5.3 La microbiota bacteriana de las fermentaciones de aceitunas verdes de mesa según PCR-DGGE

La microbiota inherente a la fermentación de aceitunas verdes de mesa al estilo Español realizada a escala industrial es diversa y compleja, tal y como se ha mostrado en el apartado anterior. No obstante, el estudio de la misma mediante técnicas dependientes de cultivo puede acarrear diversos sesgos metodológicos (Fleet, 1999) que lleven a una caracterización incompleta de la composición de la comunidad microbiana y su dinamismo real (Ampe *et al.,* 1999; Giraffa y Rossetti, 2004; Juste *et al.,* 2008). Por todo ello, los estudios de biodiversidad se complementaron, en una estrategia polifásica, con una técnica de muestreo independiente de cultivo tal como la PCR-DGGE, tal y como otros autores aconsejan (Juste *et al.,* 2008). La

PCR-DGGE es la técnica independiente de cultivo más extensamente utilizada en alimentos (Cocolin *et al.,* 2013a), especialmente en aceitunas de mesa (Abriouel *et al.,* 2011; Muccilli *et al.,* 2011; Randazzo *et al.,* 2012; Cocolin *et al.,* 2013b). Al consumir menos tiempo y esfuerzo económico que la metodología cultivo-dependiente, su uso nos ha permitido aumentar en más del 50% el número total de muestras (fermentadores) analizadas. Así, se pudo aplicar esta técnica, además de a los 20 fermentadores anteriormente analizados por técnicas dependientes de cultivo, a otros 23 fermentadores de las mismas características, repartidos entre ambos patios y procedentes de distintas zonas dentro de cada patio. Este aumento en el número de fermentadores muestreados permitió estar más en consonancia con la premisa según la cual "la biodiversidad aumenta si aumentamos el área muestreada", que establece una relación universal entre el número de especies encontradas y el tamaño de área muestreada (Rosenzweig, 1995). En este sentido, cabría esperar que empresas más pequeñas dieran lugar a productos más homogéneos con respecto a las que presentan una mayor producción. En los casos estudiados en esta Tesis Doctoral, dos patios de empresas de relativamente gran tamaño, solo pudimos detectar cierta heterogeneidad respecto a la zona de procedencia de los fermentadores en el patio 2, al emplear cebadores universales para el gen *ADNr* 16S. Este hecho podría en parte deberse a que las distintas zonas del patio 2 tienen "edades" distintas, es decir, fueron construidas y utilizadas por primera vez en campañas alejadas varios años unas de otras, por lo que la microbiota endógena pudo haber evolucionado de manera distinta.

Un factor clave, que hay que tener en cuenta cuando se utiliza una técnica independiente de cultivo, es el límite de detección de la misma. En el caso de la PCR-DGGE este límite ha sido descrito en torno a 10^3 ufc/ml o /g (Cocolin *et al.,* 2001a, 2001b), por lo que posiblemente no se hayan podido detectar algunas poblaciones minoritarias que sí habían sido identificadas anteriormente mediante las técnicas de cultivo convencionales en este mismo estudio. Este podría ser el caso de, por ejemplo, las especies de *Clostridium, Paenibacillus* y *Enterobacter*. Cada perfil de PCR-DGGE refleja la estructura de la comunidad microbiana en una muestra determinada, permitiendo la identificación de las poblaciones principales de forma semicuantitativa. Así, la información recogida en cada perfil no recoge necesariamente la riqueza total de especies microbianas de una muestra. Para evaluar si las especies identificadas son dominantes o no, por ejemplo, es preciso complementar este tipo de análisis con los resultados obtenidos en los ensayos con técnicas dependientes de cultivo (Muyzer y Smalla, 1998), como ha sido el caso de los estudios descritos en esta Tesis Doctoral.

De forma general, se pudo observar cómo tanto la fase de la fermentación como el patio de procedencia eran factores influyentes en la estructura de la comunidad microbiana. Así, la técnica PCR-DGGE, y la metodología de análisis de sus resultados, es una herramienta útil para monitorizar el curso de la fermentación de aceitunas de mesa al estilo Español. Esto se puede hacer extensible a otras fermentaciones naturales "multi-fase" complejas, como las descritas en otros alimentos como, por ejemplo, el queso o el vino. Esta metodología también permitiría evaluar si la fermentación se está llevando a cabo de forma adecuada o si se aprecian signos de posibles alteraciones antes de percibir el defecto en el producto o incluso ante de poder identificar al agente microbiano causal. Por ejemplo, en este estudio detectamos en el 10% de los fermentadores analizados la presencia de *Propionibacterium acnes* y de *Propionibacterium olivae,* especies asociadas a defectos en las aceitunas que tienen lugar al final de la fermentación o en el envasado posterior (González Cancho *et al.,* 1980; Lucena Padrós et al., 2014c).

Por otro lado, los resultados obtenidos de la identificación molecular de las bandas extraídas de los distintos perfiles de PCR-DGGE no solo corroboraron la detección de muchas de las especies bacterianas aisladas en el estudio paralelo mediante técnicas dependientes de cultivo, sino que desvelaron la presencia de algunas especies no detectadas mediante los medios de cultivo y condiciones empleados. Entre éstas últimas destacó la detección de especies pertenecientes a los géneros *Alkalibacterium, Marinilactibacillus* y *Halolactibacillus*, presentes en las muestras de una forma casi ubicua. Como ya se ha citado más arriba, las especies de estos géneros, en su conjunto, se han definido como HALAB (Ntougias, 2012), siendo la primera vez que se ha podido detectar *Alkalibacterium* y *Halolactibacillus* durante la fermentación de aceitunas de mesa. Sin embargo, pese a que mediante esta técnica independiente de cultivo se detectó el ADN de estas especies a lo largo de toda la fermentación, hay que tener en consideración la fisiología de las mismas, particularmente su intolerancia a valores de pH ácidos. Así, los resultados obtenidos mediante técnicas de cultivo, usando medios selectivos para estas especies HALAB, indicaron que la viabilidad de estas especies se limitó a la fase inicial de la fermentación. La reconocida estabilidad del ADN estaría detrás de la detección de estas especies en fases posteriores. En estos casos, el empleo de técnicas como la RT-PCR-DGGE permitiría monitorizar exclusivamente las poblaciones metabólicamente activas, tal y como han propuesto algunos autores (Cocolin *et al.,* 2013b).

5.4 Nuevas especies bacterianas aisladas de fermentaciones de aceitunas verdes de mesa

Los estudios de biodiversidad y la identificación de nuevas especies microbianas facilitan la bioprospección de un ecosistema. La biodiversidad microbiana depende de las condiciones del hábitat en cuestión, por lo que los estudios dirigidos a entornos con características significativamente diferentes, debido en muchos casos a condiciones ambientales extremas, son la mejor opción para el hallazgo de nuevos recursos microbianos, incluyendo sus genes, y compuestos biológicos de interés comercial, es decir, sus metabolitos (Bull *et al*., 1992; Jensen y Fenical 1996; Stierle *et al*., 2004, 2006). Algunos autores han aconsejado, de hecho, centrar los esfuerzos en el estudio de entornos "singulares, nuevos y altamente competitivos", en lugar de aquellos ya explorados y con un fuerte impacto de la mano del hombre para tales fines (Cao *et al*., 2009). No obstante, si tenemos en cuenta la importancia económica de los microorganismos dentro de la industria agroalimentaria, particularmente en el uso artesanal de las fermentaciones microbianas para la obtención de alimentos tradicionales, sigue siendo recomendable la búsqueda de nuevas especies o taxones en estos nichos ecológicos. Estos nuevos especímenes nos posibilitan el descubrimiento de nuevos procesos microbianos y la evaluación de su impacto en las funciones de un ecosistema conocido (Morris *et al*., 2002). Para esto último será necesario que tal descubrimiento no se base en una única muestra, sino en varias muestras independientes. Así, en el caso estudiado en esta Tesis Doctoral, se han analizado un total de 128 muestras de salmueras de aceitunas verdes de mesa elaboradas al estilo Español, teniendo en consideración tanto los estudios realizados mediante técnicas dependientes como independientes de cultivo. Estas muestras procedían de un total de 43 fermentadores diferentes, distribuidos en los patios de dos empresas distintas, y que fueron monitorizados a lo largo del tiempo coincidiendo con las tres fases de la fermentación descritas. De esta manera, el inventario de especies obtenido engloba, al menos, 12 especies de levaduras, adscritas a 7 géneros distintos, y 49 especies bacterianas, adscritas a 26 géneros diferentes. De todas estas especies se conservan cepas representativas, habiéndose analizado para ello, aproximadamente, 1250 aislados naturales.

En este contexto, estos estudios han permitido la identificación de cuatro especies bacterianas nuevas, pertenecientes a 3 géneros distintos: *Enterococcus olivae, Vibrio olivae Propionibacterium olivae* y *Propionibacterium damnosum*. Además, existe la posibilidad de que varios aislados, con características halófilas y alcalófilas y cuyo taxón más cercano es

Marinilactibacillus, pertenezcan a un nuevo género microbiano. Hay que destacar que estas nuevas especies se detectaron o bien en la fase inicial de la fermentación o bien ya en el propio envasado del producto no pasteurizado, consecuencia, probablemente, de su presencia en la fase final de la fermentación. La fisiología de estas especies estaría en consonancia con las condiciones de las salmueras en ese momento y es, precisamente, en estas fases inicial y final de la fermentación cuando microorganismos extremófilos y extremótrofos pueden jugar un papel relevante.

Así, las nuevas especies *E. olivae* y *V. olivae* pueden ser consideradas como microorganismos extremótrofos, es decir, aquellos que, si bien son capaces de soportar ciertas condiciones extremas, estas condiciones adversas nos son necesarias para su óptimo crecimiento. En el caso de estas dos especies, las condiciones de altos valores de pH y alta concentración de sal presentes en las salmueras de fermentación de las que fueron aislados no supusieron un obstáculo para el hecho de que fueran originalmente aisladas en medios de cultivo convencionales, tales como BHI-cys y MRS-BPB.

Las especies del género *Enterococcus* están ampliamente distribuidas por la naturaleza, siendo capaces de colonizar muy diversos hábitats, tales como suelos, plantas, aguas, alimentos fermentados o el tracto gastrointestinal de animales mamíferos (Devriese *et al*., 1992; Devriese y Pot, 1995; Franz *et al*., 1999; Baele *et al*., 2000; Klein, 2003). En el caso de la fermentación de aceitunas de mesa, las especies de *Enterococcus* parecen jugar un papel crucial al inicio de la fermentación, debido a su alta tolerancia a las condiciones alcalinas y también por sus características como BAL (de Castro *et al*., 2002; Corsetti *et al*., 2012). En el caso de la fermentación de aceitunas verdes al estilo Español, la mayoría de los aislados detectados de este género pertenecen a la especie *Enterococcus casseliflavus* (de Castro *et al*., 2002; De Bellis *et al*., 2010). La nueva especie *E. olivae* es fácil de diferenciar de aquellos, así como de su especie filogenéticamente más cercana, *Enterococcus saccharolyticus,* en base a caracteres fenotípicos tales como el patrón de carbohidratos fermentables, la movilidad celular y la producción de pigmentos amarillos (Devriese *et al*., 2006). Más recientemente, aislados pertenecientes a la especie *E. saccharolyticus* han sido hallados en alto número al inicio de la fermentación de aceitunas verdes de mesa al estilo Español de la variedad Hojiblanca por nuestro grupo de investigación (datos no publicados). Estos datos pertenecen a nuevos estudios de biodiversidad realizados en otras empresas aderezadoras de aceitunas de mesa en la provincia de Sevilla.

Al contrario de lo que sucede con *E. olivae*, no se ha podido encontrar hasta la fecha una relación entre la especie *Vibrio xiamenensis*, la especie filogenéticamente más cercana a *V. olivae*, y la fermentación de aceitunas de mesa. De hecho, la vinculación de especies del género *Vibrio* como colonizadoras de las fermentaciones de aceitunas de mesa es relativamente reciente (Abriouel *et al*., 2011). Más conocida es su asociación a hábitats acuáticos y, más concretamente, marinos, ya sea como individuos de vida libre o como simbiontes o parásitos de pescados, moluscos o crustáceos (Thompson *et al*., 2004). También se les ha asociado a partículas de plástico (Zettler *et al*., 2013) o a floraciones de fitoplancton (Gilbert *et al*., 2012). Los resultados descritos en esta Tesis Doctoral estarían en sintonía con las observaciones realizadas por Abriouel *et al.* (2011), esto es, la presencia de especies del género *Vibrio* no suele desarrollarse más allá de la fase inicial de la fermentación de aceitunas de mesa, a causa del pH ácido y el entorno anaerobio de los estadios más avanzados.

Por otra parte, la abundancia de aislados de la especie *V. olivae* en las salmueras durante la primera fase de la fermentación de aceitunas verdes de mesa fue similar a lo descrito para entornos marinos, esto es, del orden de 10^3-10^4 ufc/ml de agua de mar (Yooseph *et al*., 2010). Asimismo, la abundancia relativa de *Vibrio* depende fuertemente de las condiciones de crecimiento, siendo principalmente la concentración de sal y la temperatura los parámetros que correlacionan con la presencia y el dinamismo de especies de este género en un nicho microbiano concreto (Takemura *et al*., 2014). Probablemente sea la sal, habitualmente de origen marino en este sector, con la que se elaboran las salmueras, la fuente primaria de la colonización de estas especies en el entorno de la elaboración de aceituna de mesa. De todas formas, también se han descrito asociaciones de especies del género *Vibrio* con plantas (Takemura *et al*., 2014). Estas fuentes de colonización serían extensibles a otras formas de elaboración, como las aceitunas fermentadas al natural (Abriouel *et al*., 2011). Sin embargo, pueden tener una mayor relevancia en las aceitunas elaboradas al estilo Español, dado que las especies de este género están más aclimatadas a vivir en condiciones de pH neutro o ligeramente alcalino, como las que tienen lugar al inicio de la fermentación de este tipo de aceitunas.

Las nuevas especies *P. olivae* y *P. damnosum* fueron aisladas de los líquidos de gobierno procedentes de aceitunas verdes de mesa aderezadas al estilo Español, envasadas en bolsas de plástico selladas pero no pasterizadas. Estas bolsas habían sido almacenadas a temperatura ambiente antes de su comercialización y sus líquidos de gobierno presentaban signos de una

alteración no tipificada: salmueras oscuras, turbias y con olor anómalo. Las muestras procedentes de lotes distintos de estos envasados, aunque procesados en las mismas fechas, presentaron similar composición microbiana. En esta composición destacaron, por su abundancia, la presencia de aislados de estas dos nuevas especies del género *Propionibacterium.* Por otra parte, en el ensayo realizado mediante PCR-DGGE sobre las salmueras de fermentación, se detectó de nuevo la presencia, al menos, de *P. olivae,* aunque sólo en el 10% de los fermentadores analizados en la misma empresa donde se detectó esta especie en productos envasados y alterados. Por tanto, muy posiblemente esta microbiota proceda directamente de los propios frutos fermentados, pues la salmuera original es habitualmente sustituida por un líquido de gobierno en los envasados. Así, es recomendable una mayor higiene y trazabilidad del producto fermentado en las líneas de envasado para evitar la dispersión de microorganismos alterantes como los descritos.

Por otra parte, aunque este género es poco diverso, de modo que a la fecha de publicación del artículo correspondiente estaba constituido por sólo 12 especies, resulta relevante la asociación de distintas especies de este grupo con las aceitunas de mesa. Así, se ha descrito la presencia de *Propionibacterium acidipropionici* (antes *Propionibacterium pentosaceum*), *Propionibacterium jensenii* (antes *Propionibacterium zeae*) y *Propionibacterium acnes,* asociadas al defecto conocido como "zapatería" (Plastourgos y Vaughn, 1957; González Cancho *et al*., 1973, 1980). Más recientemente, *Propionibacterium microaerophilum* ha sido aislado de vertidos de la industria oleícola (Koussémon *et al*., 2001). Salvo *P. acnes,* perteneciente al grupo de especies “cutáneas” (asociadas a la piel o el intestino humano y animal), las demás pertenecen al grupo de especies “lácteas” (asociadas a ensilados y productos lácteos), y son generalmente reconocidas como microorganismos seguros e incluso beneficiosas para la salud (probióticas) (Cousin *et al*., 2011). No obstante, la capacidad de las distintas especies de este género de metabolizar el ácido láctico para producir, entre otros, ácido acético y ácido propiónico no es deseable en la fermentación de aceitunas de mesa. Esto es debido no sólo a que pueden modificar las propiedades organolépticas típicas del producto final sino "consumir" acidez libre y elevar con ello el pH de las salmueras a niveles que favorezcan el desarrollo ulterior de otras especies microbianas alterantes. Entre éstas, algunas especies del género *Clostridium,* consideradas no sólo alterantes sino incluso patógenas, pueden verse beneficiadas por estos nuevos parámetros físico-químicos en las salmueras.

Por último, como fruto de los trabajos realizados en esta Tesis Doctoral, se han obtenido una serie de aislados que, casi con toda seguridad, podrían constituir una especie nueva e incluso un género nuevo. Esta especie puede describirse como un microorganismo alcalófilo estricto y, por tanto, extremófilo. De la misma se han obtenido hasta 23 aislados naturales, distinguiéndose hasta 5 genotipos (cepas) distintos mediante RAPD-PCR (porcentaje de similitud >80%). Estos aislados proceden de las dos empresas analizadas, estando ampliamente distribuidos en las salmueras durante la primera fase de la fermentación. El grupo filogenético más cercano, en base a la homología del gen completo *ADNr* 16S, es el género *Marinilactibacillus*, por lo que inicialmente, en los artículos correspondientes, se ha denominado a estos aislados como *Marinilactibacillus* sp. Hay que reseñar que también se pudo detectar la presencia de aislados naturales pertenecientes a varias especies del género *Marinilactibacillus* que estaban extensamente distribuidos por ambos patios durante la etapa inicial de la fermentación. Otros autores han detectado la presencia de la especie *Marinilactibacillus piezotolerans* en la superficie de aceitunas de mesa tratadas con álcali (Cocolin *et al*., 2013a).

Como conclusión, la búsqueda dirigida de nuevas especies microbianas tiene un potencial biotecnológico evidente, al rescatar microorganismos con nuevos recursos genéticos y metabólicos de un ambiente natural al que ya están adaptados. Para ello, sería recomendable una estrategia combinada mediante técnicas moleculares dependientes e independientes de cultivo. Para obtener un mayor poder de resolución podría ser aconsejable aplicar, en primer lugar, una técnica independiente de cultivo potente como, por ejemplo, la pirosecuenciación, recientemente aplicada con éxito en aceitunas de mesa (Cocolin *et al*., 2013b). Posteriormente, en base a los resultados de obtenidos mediante la técnica independiente de cultivo, se elegiría una batería de medios y condiciones de cultivo especialmente diseñados, dirigidos a recuperar la máxima biodiversidad del ecosistema "*patio de fermentación de aceitunas de mesa*".

Capítulo 6
Conclusiones

1.- El estudio de la microbiota asociada a las fermentaciones de aceitunas verdes de mesa elaboradas al denominado estilo Español o sevillano ha permitido aislar, genotipar e identificar molecularmente más de 1250 aislados microbianos. Estos aislados pertenecen, al menos, a 49 especies bacterianas y 12 especies de levaduras distintas. Más de la mitad de estas especies, concretamente 34, han sido asociadas por primera vez a este tipo de fermentación de aceitunas de mesa.

2.- Esta fermentación, a pesar de las innovaciones tecnológicas introducidas en las últimas décadas, sigue siendo un proceso multifásico, microbiológicamente complejo y que implica una gran biodiversidad. Aun así, esta fermentación es fundamentalmente del tipo ácido-láctica y está dominada, en sus fases esenciales, por la especie *Lactobacillus pentosus*. La microbiota accesoria a la especie dominante es, sin embargo, muy heterogénea y su composición depende principalmente de la fase de fermentación y de las características propias de cada patio de producción.

3.- La fase inicial de la fermentación de aceitunas verdes de mesa se caracteriza por la presencia de microorganismos extremófilos y extremotrofos, capaces de crecer o tolerar, respectivamente, valores de pH muy alcalinos y concentraciones de sal moderadas (>10%). Entre los extremófilos destaca la presencia de especies del género *Vibrio, Alkalibacterium, Marinilactibacillus* y *Halolactibacillus*. Entre los que toleran estos valores destacan especies de los géneros *Aerococcus* y *Enterococcus*, así como especies del grupo *Lactobacillus plantarum*. Salvo estas últimas, las demás especies son incapaces de persistir en estadios más avanzados de la fermentación.

4.- La microbiota bacteriana característica de las fases media y final es muy parecida en cuanto a composición, estando dominadas por distintas especies de bacterias del ácido láctico pertenecientes a los géneros *Lactobacillus* y *Pediococcus* y, muy especialmente, por la especie *Lactobacillus pentosus*. Es en estas etapas en las que, por mediación de estas bacterias del ácido láctico, se alcanzan los niveles de pH y acidez libre que garantizan la estabilidad y las características del producto final.

5.- Las especies de levaduras asociadas a esta fermentación son menos abundantes y sus poblaciones alcanzan menores concentraciones que las especies bacterianas. La composición de especies es muy variable entre patios. En general, los estadios iniciales de la fermentación suelen estar dominados por especies de metabolismo eminentemente

fermentativo tales como *Issatchenkia orientalis, Candida tropicalis* o *Saccharomyces cerevisiae*. En cambio, en las fases avanzadas de esta fermentación son características las especies *Candida thaimueangensis, Pichia manshurica/membranifaciens* o *Rhodotorula mucilaginosa*, todas ellas con metabolismo oxidativo.

6.- El genotipado de los aislados microbianos mediante RAPD-PCR y el consecuente análisis de los dendrogramas filogenéticos resultantes han permitido comprobar que la sucesión microbiana se da también al nivel de cepas a lo largo de la fermentación. Por otra parte, la presencia de genotipos microbianos que podríamos denominar "cosmopolitas" pone de manifiesto la presencia de cepas bien adaptadas a la ecología de este tipo de fermentaciones en la región.

7.- Los resultados obtenidos mediante análisis independiente de cultivo utilizando la técnica PCR-DGGE han confirmado, casi en su totalidad, los obtenidos mediante las técnicas de cultivo microbiológico. Con esta técnica se han podido obtener huellas genéticas específicas de las comunidades microbianas características de cada patio y de cada fase de la fermentación. Estos análisis han servido también para evidenciar la presencia de grupos microbianos no detectados previamente por las técnicas dependientes de cultivo, como son algunas especies bacterianas halófilas y alcalófilas pertenecientes a los géneros *Alkalibacterium* y *Halolactibacillus*, detectadas por primera vez durante la fermentación de aceitunas de mesa.

8.- Especies del grupo de bacterias del ácido láctico halófilas y alcalófilas, recientemente descritas y denominadas HALABs, destacan por su ubicuidad y porque parecen jugar un papel crucial al inicio de la fermentación. La diversidad, abundancia y persistencia de estas especies se ve muy condicionada por las condiciones de procesamiento de los frutos durante las operaciones previas a la fermentación. Especies pertenecientes a los tres géneros descritos hasta la fecha de HALABs han sido aisladas en este estudio, a saber, *Marinilactibacillus*, *Alkalibacterium* y *Halolactibacillus*.

9.- A partir de este estudio se han aislado y descrito cuatro nuevas especies bacterianas: *Enterococcus olivae, Propionibacterium olivae, Propionibacterium damnosum* y *Vibrio olivae.* Por otra parte, el análisis de la secuencia del gen *ADNr* 16S de un grupo de aislados indica que podría tratarse de, al menos, una nueva especie bacteriana, cuya mayor homología la manifiesta frente al género *Marinilactibacillus*.

CAPÍTULO 7
OTRAS APORTACIONES CIENTÍFICAS

Los trabajos desarrollados por la autora de esta Tesis Doctoral han dado lugar también a la siguiente relación de comunicaciones a congresos y reuniones científicas:

Autores: José Luis Ruiz Barba, Antonio Maldonado Barragán, Helena Lucena Padrós, Belén Caballero Guerrero y Asunción Forniés Duerto.
Título: Biotecnología de Bacterias Lácticas.
Tipo de participación: asistencia y póster.
Congreso: XXXIV Asamblea del Instituto de la Grasa. Sevilla.
Publicación: Libro de resúmenes.
Lugar celebración: Sevilla.
Fecha: 2010

Autores: Helena Lucena Padrós, Belén Caballero Guerrero, José Luis Ruiz Barba y Antonio Maldonado Barragán.
Título: Biodiversity in Spanish-style Green Olive Fermentations: a non-explored microbial niche with wide potential applications.
Tipo de participación: Asistencia y póster.
Congreso: IV Jornadas Internacionales de la Aceituna de Mesa.
Publicación: libro de resúmenes, páginas 130-131.
Lugar celebración: Córdoba.
Fecha: 16-17 de Febrero de 2012.

Autores: Antonio Maldonado Barragán, Belén Caballero Guerrero, Helena Lucena Padrós and José Luis Ruiz Barba
Título: Unveiling the genome of *Lactobacillus pentosus* IG1, a strain isolated from Spanish-style green olive fermentations: great adaptability with biotechnological and probiotic properties.
Tipo de participación: asistencia y póster
Congreso: IV Jornadas Internacionales de la Aceituna de Mesa.
Publicación: libro de resúmenes. Páginas 128-129.
Lugar celebración: Córdoba
Fecha: 16-17 de Febrero de 2012

Autores: Helena Lucena Padrós, Belén Caballero Guerrero, José Luis Ruiz Barba y Antonio Maldonado Barragán.

Título: Biodiversidad microbiana en fermentaciones de aceitunas verdes de mesa al Estilo Español.
Tipo de participación: asistencia y comunicación oral.
Congreso: VI Reunión de la red temática BAL: Participación de las bacterias lácticas en la salud humana y en la calidad alimentaria.
Publicación: libro de resúmenes, página 32.
Lugar celebración: Tarragona.
Fecha: 28-29 de Junio 2012.

Autores: Helena Lucena Padrós, Belén Caballero Guerrero, Esther Jiménez, Juan Miguel Rodríguez, Antonio Maldonado Barragán y José Luis Ruiz Barba.
Título: Estudio de la diversidad bacteriana en fermentaciones de aceitunas verdes de mesa mediante la técnica independiente de cultivo DGGE.
Tipo de participación: asistencia y comunicación oral.
Congreso: VII Reunión de la Red Temática BAL: participación de las bacterias lácticas en la salud humana y en la calidad alimentaria.
Publicación: libro de resúmenes, página 12.
Lugar celebración: Madrid.
Fecha: 4-5 de Julio 2013

Autores: Helena Lucena Padrós, Belén Caballero Guerrero, Antonio Maldonado Barragán y José Luis Ruiz Barba.
Título: Diversidad genética y dinamismo de las poblaciones microbianas asociadas a fermentaciones de aceitunas verdes de mesa al estilo "Español" en empresas de gran tamaño.
Tipo de participación: Asistencia y Comunicación oral.
Congreso: VIII Reunión de la Red Temática BAL: participación de las bacterias lácticas en la salud humana y en la calidad alimentaria.
Publicación: libro de resúmenes, página 30.
Lugar celebración: San Adrián, Navarra.
Fecha: 25-27 de Junio 2014

Autores: Helena Lucena Padrós, Belén Caballero Guerrero, Esther Jiménez, Juan Miguel Rodríguez, Antonio Maldonado Barragán y José Luis Ruiz Barba.
Título: Análisis de la diversidad microbiana asociada a la fermentación de aceitunas de mesa mediante técnicas dependientes e independientes de cultivo.

Tipo de participación: asistencia y póster.
Congreso: III Congreso Científico de Investigadores en Formación en Agroalimentación ceiA3.
Publicación: asistencia y póster.
Lugar celebración: Córdoba.
Fecha: 18-19 de Noviembre 2014

Autores: Helena Lucena Padrós y José Luis Ruiz Barba.
Título: Aislamiento, diversidad y evolución de bacterias lácticas halófilas y alcalófilas (HALAB) asociadas a fermentaciones de aceitunas verdes de mesa al estilo "Español".
Congreso: IX Reunión de la Red Temática BAL: participación de las bacterias lácticas en la salud humana y en la calidad alimentaria.
Publicación: libro de resúmenes, página 26.
Lugar celebración: Madrid.
Fecha: 29-30 de Junio 2015

Autores: Helena Lucena Padrós, Antonio Maldonado Barragán y José Luis Ruiz Barba.
Título: Evaluación del potencial probiótico y biotecnológico de la biodiversidad microbiana asociada a la fermentación de aceitunas verdes de mesa.
Congreso: VII Workshop Probióticos, Prebióticos y Salud: Evidencia Científica
Publicación: libro de resúmenes.
Lugar celebración: Sevilla.
Fecha: 28-29 de Enero 2016

Además, durante el desarrollo de la presente Tesis Doctoral, la autora ha participado en los trabajos experimentales que han dado lugar a la elaboración de tres publicaciones científicas adicionales, a saber:

- **Maldonado-Barragán, A., Caballero-Guerrero, B., Lucena-Padrós, H., Ruiz-Barba, J.L. 2011.** Genome sequence of *Lactobacillus pentosus* IG1, a strain isolated from Spanish-style green olive fermentations. Journal of Bacteriology 193, 5605.

- **Maldonado-Barragán, A., Caballero-Guerrero, B., Lucena-Padrós, H., Ruiz-Barba, J.L. 2013.** Induction of bacteriocin production by coculture is widespread

among plantaricin-producing *Lactobacillus plantarum* strains with different regulatory operons. Food Microbiology 33, 40-47.

- **Caballero-Guerrero, B., Lucena-Padrós, H., Maldonado-Barragán, A., Ruiz-Barba J.L. 2013.** High-salt brines compromise autoinducer-mediated bacteriocinogenic *Lactobacillus plantarum* survival in Spanish-style green olive fermentations. Food Microbiology 33, 90-96.

Finalmente, la autora de esta Tesis Doctoral, durante el transcurso de la misma, ha participado en los siguientes Proyectos de Investigación:

1. **Proyecto de Investigación:** " *Calidad y seguridad en fermentaciones de aceitunas de mesa: cultivos iniciadores, microbiota asociada y biofilms*".
   Código: AGL2009-07861.
   Investigador Principal: José Luis Ruiz Barba
   Financiado por: Ministerio de Ciencia e Innovación.
   Duración: Enero de 2010 a Diciembre de 2012.

2. **Proyecto de Investigación:** " *Caracterización de la microbiota de las fermentaciones de aceitunas verdes en Andalucía mediante técnicas moleculares*".
   Código: AGR2009-04621.
   Investigador Principal: Antonio Maldonado Barragán
   Financiado por: Programa de Proyectos de Excelencia de la Junta de Andalucía.
   Duración: Febrero de 2010 a Febrero de 2012.

3. **Proyecto de Investigación:** "*Recursos microbianos asociados a las fermentaciones de aceitunas de mesa: explotación biotecnológica de una biodiversidad natural poco explorada*".
   Código: AGL2012-33400.
   Investigador Principal: José Luis Ruiz Barba
   Financiado por: el Ministerio de Economía y Competitividad.
   Duración: Enero de 2013 a Diciembre de 2015.

4. **Proyecto de Investigación:** *"Explotación de la biodiversidad microbiana asociada a las fermentaciones de aceitunas de mesa en Andalucía: probióticos, genómica y biotecnología"*.
   Código: AGR2011-7345.

Investigador Principal: José Luis Ruiz Barba
Financiado por: Programa de Proyectos de Excelencia de la Junta de Andalucía.
Duración: Mayo de 2013 a Septiembre de 2016.

5. **Proyecto de Investigación:** *"Análisis microbiológico y físico-químico de salmueras de fermentaciones tumultuosas de aceitunas verdes de mesa"* (FERMENTAOLIVA)
Código: IDI-20131247.
Investigador Principal: José Luis Ruiz Barba
Financiado por: ESTEPAOLIVA S.L., a través de CDTI.
Duración: Noviembre de 2013 a Junio de 2015.

6. **Proyecto de Investigación:** *"Análisis microbiológico y físico-químico de salmueras de fermentaciones tumultuosas de aceitunas verdes de mesa"* (FERMENTAOLIVA)
Código: IDI-20131248.
Investigador Principal: José Luis Ruiz Barba
Financiado por: GESTOLIVA S.A., a través de CDTI.
Duración: Noviembre de 2013 a Junio de 2015.

Capítulo 8
Bibliografía

- **Abdelkafi, S., Labat, M., Casalot, L., Chamkha, M. y Sayadi, S., 2006.** Isolation and characterization of *Halomonas* sp. strain IMPC, a p-coumaric acid-metabolising bacterium that decarboxylates other cinnamic acids under hypersaline conditions. FEMS Microbiology Letters 255, 108-114.

- **Abriouel, H., Benomar, N., Lucas, R. y Gálvez, A., 2011.** Culture-independent study of the diversity of microbial populations in brines during fermentation of naturally-fermented Aloreña green table olives. International Journal of Food Microbiology 144, 487-496.

- **Alexopoulos, C.J., Mims, C.W. y Blackwell, M., 1996.** Introductory mycology. John Wiley & Sons, New York. New York, USA.

- **Ampe, F., ben Omar, N., Moizan, C., Wacher, C. y Guyot, J.P., 1999.** Polyphasic study of the spatial distribution of microorganisms in Mexican pozol, a fermented maize dough, demonstrates the need for cultivation-independent methods to investigate traditional fermentations. Applied and Environmental Microbiology 65, 5464-5473.

- **Aponte, M., Ventorino, V., Blaiotta, G., Volpe, G., Farina, V., Avellone, G., Lanza, C.M. y Moschetti, G., 2010**. Study of green Sicilian table olive fermentations through microbiological, chemical and sensory analyses. Food Microbiology 27, 162-170.

- **Aponte, M., Blaiotta, G., La Croce, F., Mazzaglia, A., Farina, V., Settanni, L. y Moschetti, G., 2012**. Use of selected autochthonous lactic acid bacteria for Spanish style table olive fermentation. Food Microbiology 30, 8-16.

- **Arroyo-López, F.N., 2006.** Conservación y envasado de aceitunas de mesa "aliñadas" de la variedad Manzanilla-Aloreña. Tesis Doctoral, Universidad de Sevilla.

- **Arroyo-López, F.N., Durán-Quintana, M.C., Ruiz-Barba, J.L., Querol, A. y Garrido-Fernández, A., 2006.** Use of molecular methods for the identification of yeast associated with table olives. Food Microbiology 23, 791-796.

- **Arroyo-López, F.N., Querol, A., Bautista-Gallego, J. y Garrido-Fernández, A., 2008.** Role of yeasts in table olive production. International Journal of Food Microbiology 128, 189-196.

- **Arroyo-López, F.N., Romero-Gil, V., Bautista-Gallego, J., Rodríguez-Gómez, F., Jiménez-Díaz, R., *et al.*, 2012a.** Yeasts in table olive processing: desirable or spoliage microorganisms? International Journal of Food Microbiology 160, 42-49.

- **Arroyo-López, F.N., Bautista-Gallego, J., Domínguez-Manzano, J., Romero-Gil, V., Rodriguez-Gómez, F., García-García, P., *et al*., 2012b.** Formation of lactic acid bacteria-yeasts communities on the olive surface during Spanish- style Manzanilla fermentations. Food Microbiology 32, 295-301.

- **ASEMESA (Asociación de Exportadores e Industriales de Aceitunas de Mesa), 2014.** Datos generales del sector de la aceituna de mesa. http://www.asemesa.es/content/datos_generales_del_sector. Acceso en 01.12.14.

- **Atlas, R. y Bartha, R., 2002**. Ecología microbiana y Microbiología Ambiental. IV Edición. España, Addison Wesley. pp 677.

- **Baele, M., Baele, P., Vaneechoutte, M., Storms, V., Butaye, P., Devriese, L.A., Verschraegen, G., Gillis, M. y Haesebrouck, F., 2000**. Application of tRNA intergenic spacer PCR for identification of *Enterococcus* species. Journal of Clinical Microbiology 38, 4201-4207.

- **Bartkowski, B., Lienhoop, N. y Hansjürgens, B., 2015.** Capturing the complexity of biodiversity: a critical review of economic valuation studies of biological diversity. Ecological Economics 113, 1-14.

- **Barranco, D., 2008**. Variedades y patrones. En: El cultivo del olivo. Barranco, D., Fernández-Escobar, R. y Rallo, L. (eds.). 6ª Ed. Mundi-Prensa y Junta de Andalucía. Madrid España, pps. 64-92.

- **Barranco, D., Rallo, L. y Fernández-Escobar, R., 2004**. El cultivo del olivo. Ediciones Mundi-Prensa.

- **Battcock, M. y Azam-Ali, S., 1998.** Bacterial fermentations. In: Fermented Fruits and Vegetables. A Global Perspective. FAO Agricultural Services. Bulletin num. 134.

- **Bautista-Gallego, J., Rodríguez-Gómez, F., Barrio, E., Querol, A., Garrido-Fernández, A. y Arroyo-López, F.N., 2011.** Exploring the yeast biodiversity of Green table olive industrial fermentations for technological applications. International Journal of Food Microbiology 147, 89-96.

- **Benito, M.J., Martín, A., Aranda, E., Pérez-Nevado, F., Ruiz-Moyano, S. y Córdoba, M.G., 2007.** Characterization and selection of autochthonous lactic acid bacteria isolated from traditional Iberian dry-fermented salchichón and chorizo sausages. Journal of Food Science 72, 193-201.

- **Bisby, F.A., Coddington, J., Thorpe, J.P., *et al*., 1995.** Characterization of biodiversity. En: Global Biodiversity Assessment (eds. Heywood VH, Gardener K). pp. 22-106, Cambridge University Press for UNEP, Cambridge.

- **Blaiotta, G., Moschetti, G., Simeoli, E., Andolfi, R., Villani, F. y Coppola, S., 2001.** Monitoring lactic acid bacteria strains during "Cacioricotta" cheese production by restriction endonucleases analysis and pulsed-field gel electrophoresis. The Journal of Dairy Research 68, 139-144.

- **Boekhout, T., Fonseca, Á., Sampaio, J.P., Bandoni, R.J., Fell, J.W. y Kwon-Chung, K.J., 2011.** Discussion of teleomorphic and anamorphic basidiomycetous yeasts. In: Kurtzman, C.P., Fell, J.W., Boekhout, T. (Eds.). The Yeasts, a Taxonomic Study, 5th ed. Elsevier, Amsterdam, pp. 1339-1372.

- **Borbolla y Alcalá, J.M.R., *et al*., 1956.** El aderezo de aceitunas verdes. Consejo Superior de Investigaciones Científicas. Madrid.

- **Borbolla y Alcalá, J.M.R., Fernández-Díez, M.J. y González-Cancho, F., 1969.** Influence of pasteurization and lye treatment on the fermentation of Spanish-style Manzanilla olives. Applied Microbiology 17, 734-736.

- **Borbolla y Alcalá, J.M.R. y González Cancho, F., 1975.** Preparación de aceitunas verdes. Ponencia al II Seminario Oleícola Internacional, Córdoba, España.

- **Borbolla y Alcalá, J.M.R. y Rejano, L., 1979.** Sobre la preparación de la aceituna estilo sevillano. La fermentación I. Grasas y Aceites 30, 175-185.

- **Borbolla y Alcalá, J.M.R. y Rejano, L., 1981.** Sobre la preparación de la aceituna estilo sevillano. El tratamiento con lejía. Grasas y Aceites 32, 181-189.

- **Botta, C. y Cocolin, L., 2012**. Microbial dynamics and biodiversity in table olive fermentation: culture-dependent and-independent approaches. Frontiers in Microbiology 3:245. Doi: 10.3389/fmicb.2012.00245.

- **Brenner, D.J., Staley, J.T. y Krieg, N.R., 2001.** Classification of procaryotic organisms and the concept of bacterial speciation, in: 2nd ed, in: D.R. Boone, R.W. Castenholz, G.M. Garrity (Eds.), Bergey's Manual of Systematic Bacteriology, Vol. 1, Springer, New York, USA, pp. 27-31.

- **Buckenhüskes, H.J., 1993**. Selection criteria for lactic acid bacteria to be used as starter cultures. FEMS Microbiology Reviews 12, 253-272.

- **Buée, M., Reich, M., Murat, C., Morin, E., Nilsson, R., Uroz, S. y Martin, F., 2009.** 454 Pyrosequencing analyses of forest soils reveal an unexpectedly high fungal diversity. New Phytologist 2, 449-456.

- **Bull, A.T., Goodfellow, M. y Slater, J.H., 1992.** Biodiversity as a source of innovation in biotechnology. Annual Review of Microbiology 46, 219-252.

- **Camu, N., De Winter, T., Verbrugghe, K., Cleenwerck, I., Vandamme, P., Takrama, J.S., Vancanneyt, M. y De Vuyst, L., 2007.** Dynamics and Biodiversity of Populations of Lactic Acid Bacteria and Acetic Acid Bacteria Involved in Spontaneous Heap Fermentation of Cocoa Beans in Ghana. Applied and Environmental Microbiology 73, 1809-1824.

- **Cao, Y., Jiang, Y. y Hua, X.L., 2009.** Great Shangri-La composition analysis of soil actinomycetes and biological activity determination. Acta Microbiologica Sinica 49, 105-109.

- **Capozzi, V., Russo, P., Beneduce, L., Weidmann, S., Grieco, F., Guzzo, J. y Spano, G., 2010.** Technological properties of *Oenococcus oeni* strains isolated from typical southern Italian wines. Letters in Applied Microbiology 50, 327-334.

- **Casaregola, S., Jacques, N., Louis-Mondesir, C., Coton, M. y Coton, E., 2013.** *Citeromyces nyonsensis* sp. nov., a novel yeast species isolated from black olive brine. International Journal of Systematic and Evolutionary Microbiology 63, 3086-3090.

- **CBD (Convenio sobre la Diversidad Biológica), 1992.** The United Nations Convention on Biological Diversity. Reprinted in International Legal Materials 31 (5 June 1992): 818. (Entered into force 29 December 1993).

- **Chalmers, N.R., 1996**. Monitoring and inventoring biodiversity. Collections data and training, En: F. Di Castri y T. Yuonés (Eds.). Biodiversity, science and development. Cambridge: CAB International, University Press. pp. 171-179.

- **Christensen, H., Bisgaard, M., Frederiksen, W., Mutters, R., Kuhnert, P. y Olsen, J.E., 2001.** Is characterization of a single isolate sufficient for valid publication of a new genus or species? Proposal to modify Recommendation 30b of the Bacteriological Code (1990 Revision). International Journal of Systematic and Evolutionary Microbiology 51, 2221-2225.

- **Chun, J., *et al*., 2007.** EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. International Journal of Systematic and Evolutionary Microbiology 57, 2259-2261.

- **Cocolin, L., Manzano, M., Cantoni, C. y Comi, G., 2001a**. Denaturing gradient gel electrophoresis analysis of the 16S *rRNA* gene V1 region to monitor dynamic changes in the bacterial population during fermentation of Italian sausages. Applied and Environmental Microbiology 67, 5113-5121.

- **Cocolin, L., Manzano, M., Aggio, D., Cantoni, C. y Comi, G., 2001b**. A novel polymerase chain reaction (PCR) denaturing gradient gel electrophoresis (DGGE) for the identification of *Micrococcaceae* strains involved in meat fermentations. Its application to naturally fermented Italian sausages. Meat Science 57, 59-64.

- **Cocolin, L., Dolci, P. y Rantsiou, K., 2011**. Biodiversity and dynamics of meat fermentations: the contribution of molecular methods for a better comprehension of a complex ecosystem. Meat Science 89, 296-302.

- **Cocolin, L., Alessandria, V., Dolci, P., Gorra, R. y Rantsiou, K., 2013a.** Culture independent methods to assess the diversity and dynamics of microbiota during food fermentation. International Journal of Food Microbiology 167, 29-43.

- **Cocolin, L., Alessandria, V., Botta, C., Gorra, R., De Filippis, F., Ercolini, D. y Rantsiou, K., 2013b.** NaOH-debittering induces changes in bacterial ecology during table olives fermentation. PLoS One 8 (7), e69074. http://dx.doi.org/10.1371/journal.pone.0069074

- **Colwell, R.R., 1970.** Polyphasic taxonomy of the genus *Vibrio*: numerical taxonomy of *Vibrio cholerae, Vibrio parahaemolyticus*, and related strains. Journal of Bacteriology 104, 410-433.

- **COI (Consejo Oleícola Internacional), 1981.** Norma cualitativa unificada aplicable a las aceitunas de mesa en el comercio internacional T/OT/Doc. nº 15 de 2 de octubre de 1980 revisada en 1981. Resolución Nº RES-3/43-III/80.

- **COI (Consejo Oleícola Internacional), 2004**. Norma Comercial Aplicable a las Aceitunas de Mesa. Resolución Nº RES-2/91-IV/04.

- **COI (Consejo Oleícola Internacional), 2014.** Cifras Aceitunas de Mesa Mundial y Europea. Disponible Online en: http://www.internationaloliveoil.org/ Acceso en 01.12. 2014

- **COI (Consejo Oleícola Internacional), 2015.** Market newsletter, Mercado Oleícola Nº 92- Marzo 2015. Disponible Online en: http://www.internationaloliveoil.org/

- **Cordero-Bueso, G., Arroyo, T., Serrano, A., Tello, J., Aporta, I., Vélez, M.D. y Valero, E., 2011**. Influence of the farming system and vine variety on yeast communities associated with grape berries. International Journal of Food Microbiology 145, 132-139.

- **Corsetti A., Perpetuini, G., Schirone, M., Tofalo, R. y Suzzi G., 2012**. Application of starter cultures to table olive fermentation: an overview on the experimental studies. Frontiers in Microbiology 3:248. doi: 10.3389/fmicb.2012.00248

- **Coton, E., Coton, M., Levert, D., Casaregola, S. y Sohier, D., 2006.** Yeast ecology in French cider and black olive natural fermentations. International Journal of Food Microbiology 108, 130-135.

- **Cousin, F.J., Mater, D.D.G., Foligné, B. y Jan, G., 2011.** Dairy propionibacteria as human probiotics: a review of recent evidence. Dairy Science & Technology 91, 1-26.

- **Cowan, S.T., 1968.** A dictionary of microbial taxonomic usage. Oliver & Boyd Eds., Edinburgh.

- **Csoma, H., Zakany, N., Capece, A., Romano, P. y Sipiczki, M., 2010.** Biological diversity of *Saccharomyces* yeasts of spontaneously fermenting wines in four wine regions: comparative genotypic and phenotypic analysis. International Journal of Food Microbiology 140, 239-248.

- **Curtis, T.P., Sloan, W.T. y Scannell, J.W., 2002.** Estimating prokaryotic diversity and its limits. Proceedings of the National Academy of Sciences of the United States of America 99, 10494-10499.

- **Dar, S.A., Yao, L., van Dongen, U., Kuenen, J.G. y Muyzer, G., 2007**. Analysis of diversity and activity of sulfate-reducing bacterial communities in sulfidogenic bioreactors using 16S *rRNA* and *dsrB* genes as molecular markers. Applied and Environmental Microbiology 73, 594-604.

- **De Angelis, M., de Candia, S., Calasso, M.P., Faccia, M., Guinee, T.P., Simonetti, M.C. y Gobbetti, M., 2008**. Selection and use of autochthonous multiple strain cultures for the manufacture of high-moisture traditional Mozzarella cheese. International Journal of Food Microbiology 125, 123-132.

- **De Bellis, P., Valerio, F., Sisto, A., Lonigro, S.L. y Lavermicocca, P., 2010.** Probiotic table olives: microbial populations adhering on olive surface in fermentation sets inoculated with the probiotic strain *Lactobacillus paracasei* IMPC2.1 in an industrial plant. International Journal of Food Microbiology 140, 6-13.

- **De Castro, A., Montaño, A., Casado, F.J., Sánchez, A.H. y Rejano, L., 2002.** Utilization of *Enterococcus casseliflavus* and *Lactobacillus pentosus* as starter culture for Spanish-style green olive fermentation. Food Microbiology 19, 637-644.

- **Devriese, L.A., Collins, M.D. y Wirth, R., 1992.** The genus *Enterococcus*. En: The Prokaryotes: A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications, pp. 1465-1481. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K. H. Schleifer. New York: Springer.

- **Devriese, L.A. y Pot, B., 1995.** The genus *Enterococcus*. En: The Genera of Lactic Acid Bacteria, pp. 327-367. Edited by B. J. B. Wood & W. H. Holzapfel. London: Blackie Academic & Professional.

- **Devriese, L., Baele, M. y Butaye, P., 2006.** The genus *Enterococcus*: Taxonomy. En: The Prokaryotes: a Handbook on the Biology of Bacteria. vol. 4; 163-174. 3rd edn. Edited by M. Dworkin, S. Falkow, E. Rosenberg, K-H. Schleifer and Stackebrandt, E. New York: Springer.

- **Domínguez-Manzano, J., Olmo-Ruiz, C., Bautista-Gallego, J., Arroyo- López, F.N., Garrido-Fernández, A. y Jiménez-Díaz, R., 2012.** Biofilm formation on abiotic and biotic surfaces during Spanish style green table olive fermentation. International Journal of Food Microbiology 157, 230-238.

- **Doulgeraki, A.I., Hondrodimou, O., Eliopoulos, V. y Panagou, E., 2012.** Lactic acid bacteria and yeast heterogeneity during aerobic and modified atmosphere packaging storage of natural black Conservolea olives in polyethylene pouches. Food Control 26, 49-57.

- **Doulgeraki, A., Pramateftaki, P., Argyri, A., Nychas, G.-J.E., Tassou, C.C. y Panagou, E.Z., 2013.** Molecular characterization of lactic acid bacteria isolated from industrially fermented Greek table olives. LWT-Food Science and Technology 50, 353-356.

- **Durán-Quintana, M.C., Romero, C., García, P., Brenes, M. y Garrido, A., 1997.** Bacterias del ácido láctico en la fermentación de aceitunas de mesa. Grasas y Aceites, 48, 297-311.

- **Eilers, H., Pernthaler, J., Glöckner, F.O. y Amann, R., 2000.** Culturability and in situ abundance of pelagic bacteria from the North Sea. Applied and Environmental Microbiology 66, 3044-3051.

- **Ercolini, D., Hill, P.J. y Dodd, C.E.R., 2003.** Bacterial Community Structure and Location in Stilton Cheese. Applied and Environmental Microbiology 69, 3540-3548.

- **Ercolini, D., Villani, F., Aponte, M. y Mauriello, G., 2006**. Fluorescence *in situ* hybridization detection of *Lactobacillus plantarum* group on olives to be used in natural fermentations. International Journal of Food Microbiology 112, 291-296.

- **Ercolini, D., Frisso, G., Mauriello, G., Salvatore, F. y Coppola, S., 2008.** Microbial diversity in natural whey cultures used for the production of Caciocavallo Silano PDO cheese. International Journal of Food Microbiology 124, 164-170.

- **Esteve-Zarzoso, B., Belloch, C., Uruburu, F. y Querol, A., 1999.** Identification of yeasts by RFLP analysis of the 5.8S *rRNA* gene and the two ribosomal internal transcribed spacers. International Journal of Systematic Bacteriology 49, 329-337.

- **Estrada Cabezas, J., 2011**. La aceituna de mesa: nociones sobre sus características, elaboración y cualidades. Aceituna de Mesa, Fundación para el fomento y promoción y Diputación de Sevilla.

- **ESYRCE (Encuesta sobre Superficie y Rendimientos de cultivos), 2013.** Encuesta sobre Superficie y Rendimientos de cultivos. Subdirección General Estadísticas Agroalimentarias, SGT. Ministerio de Medio Ambiente y Medio Rural y Marino. Pps.5 de 44.

- **Fell, J.W., Boekhout, T., Fonseca, Á., Scorzetti, G. y Statzell-Tallman, A., 2000.** Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. International Journal of Systematic and Evolutionary Microbiology 50, 1351-1371.

- **Felske, A., Wolterink, A., van Lis, R., de Vos, W.M. y Akkermans, A.D.L., 1999.** Searching for the predominant soil bacteria: 16S *rDNA* cloning versus strain cultivation. FEMS Microbiology Ecology 30, 137-145.

- **Fernández-Díez, M.J., 1983.** Olives. In H.-J. Rehm, & G. Reed (Eds.), Biotechnology, pp. 379-397. Weinheim, Germany: VCH.

- **Fernández-Díez, M.J., *et al*., 1985**. Biotecnología de la aceituna de mesa. Consejo Superior de Investigaciones Científicas, Madrid.

- **Fischer, S.G. y Lerman, L.S., 1983.** DNA fragments differing by single basepair substitutions are separated in denaturing gradient gels: correspondence with melting theory. Proceedings of the National Academy of Sciences of the United States of America 80, 1579-1583.

- **Fleet, G.H., 1999.** Microorganisms in food ecosystems. International Journal of Food Microbiology 50, 101-117.

- **Franz, C.M.A.D., Holzapfel, W.H. y Stiles, M.E., 1999.** Enterococci at the crossroads of food safety? International Journal of Food Microbiology 47, 1-24.

- **Gala, E., Landi, S., Solieri, L., Nocetti, M., Pulvirenti, A. y Giudici, P., 2008.** Diversity of lactic acid bacteria population in ripened Parmigiano Reggiano cheese. International Journal of Food Microbiology 125, 347-351.

- **Garrido-Fernández, A., *et al*., 1995.** Olive fermentations. En: H.-J. Rehm and G. Reed (ed.), Biotechnology: Enzymes, Biomass, Food and Feed. VCH Inc, NY.

- **Garrido-Fernández, A., Fernández-Díez, M.J. y Adams, M.R., 1997.** Table olives, production and processing. Champman and Hall, London. pp. 134-197.

- **Ghebremedhin, B., Layer, F., König, W. y König, B., 2008.** Genetic classification and distinguishing of *Staphylococcus* species based on different partial gap, 16S *rRNA, hsp60, rpoB, sodA*, and *tuf* gene sequences. Journal of Clinical Microbiology 46, 1019-1025.

- **Gilbert, J.A., Steele, J.A., Caporaso, J.G., Steinbrück, L., Reeder, J., Temperton, B., *et al*., 2012**. Defining seasonal marine microbial community dynamics. The ISME Journal 6, 298-308.

- **Giraffa, G. y Rossetti, L., 2004**. Monitoring of the bacterial composition of dairy starter cultures by RAPD-PCR. FEMS Microbiology Letters 237, 133-138.

- **González, J. M. y Saiz-Jimenez, C., 2002**. A fluorimetric method for the estimation of G+C mol% content in microorganisms by thermal denaturation temperature. Environmental Microbiology 4, 770–773.

- **González, J. M. y Saiz-Jimenez, C., 2005**. A simple fluorimetric method for the estimation of DNA-DNA relatedness between closely related microorganisms by thermal denaturation temperatures. Extremophiles 9, 75–79.

- **González Cancho, F., Fernández Díez, M.J., Nosti Vega, M. y Buzcu, N., 1973.** Caractéristiques des principaux microorganismes responsables de la ‘‘zapateria’’. Informe Oléicoles International 60-61, 133-144.

- **González Cancho, F., Rejano, L. y Borbolla y Alcalá, J.M.R., 1980.** La formación de ácido propiónico durante la conservación de las aceitunas verdes de mesa. III. Microorganismos responsables. Grasas Aceites 31, 245-250.

- **González Cancho, F. y Durán Quintana, M.C., 1981.** Bacterias cocáceas del ácido láctico en el aderezo de aceitunas verdes. Grasas Aceites 32, 373-379.

- **González Cancho, F., Rejano Navarro, L., Duran Quintana, M.C, Sánchez Roldan, F., García García, R, Castro Gómez-Millán, A. y Garrido Fernández, A., 1983.** Influencia de la adición de HCI en la fermentación de aceitunas verdes estilo español. Grasas y Aceites 34, 375-379.

- **Goodfellow, M. y O´Donnell, A.G., 1993**. Roots of bacterial systematics. In: Handbook of New Bacterial Systematics, pp. 3-54. Goodfellow M y O'Donnell AG (Eds.). London: Academic Press.

- **Goodman, R.M., Speers, M.A., Mcleroy, K., *et al*., 1998**. Identifying and defining the dimensions of community capacity to provide a basis for measurement. Health Education & Behavior, 25, 258-278.

- **Grounta, A., Doulgeraki, A.I. y Panagou, E.Z., 2015**. Quantification and characterization of microbial biofilm community attached on the surface of fermentation vessels used in green table olive processing. International Journal of Food Microbiology 203, 41– 48.

- **Gullo, M. y Giudici, P., 2008.** Acetic acid bacteria in traditional balsamic vinegar: phenotypic traits relevant for starter cultures selection. International Journal of Food Microbiology 125, 46-53.

- **Hariston, N.G., Allan, J.D., Colwell, R.K., Futuyma, D.J., Howell, J., Lubin, M.D., Mathias, J. y Vandermeer, J.H., 1968**. The relationship between species diversity and stability: an experimental approach with protozoa and bacteria. Ecology 49, 1091-1101.

- **Harpole, W., 2010**. Neutral theory of species diversity. Nature Education Knowledge 1: 31.

- **Hawksworth, D.L., Crous, P.W., Redhead, S.A., Reynolds, D.R., Samson, R.A., *et al*., 2011.** The Amsterdam Declaration on Fungal Nomenclature. IMA Fungus 2, 105-112.

- **Hawksworth, D.L., 2012.** Managing and coping with names of pleomorphic fungi in a period of transition. IMA Fungus 4, 53-56.

- **Hebert, P.D.N., Cywinska, A., Ball, S.L. y DeWaard, J.R., 2003.** Biological identifications through DNA barcodes. Proceedings of the Royal Society of London B, Biological Sciences 270, 313-321.

- **Heperkan, D., 2013**. Microbiota of table olive fermentations and criteria of selection for their use as starters. Frontiers in Microbiology 4:143. doi: 10.3389/fmicb.2013.00143

- **Hibbett, D.S., Binder, M., Bischoff, J.F., Blackwell, M., Cannon, P.F., *et al*., 2007.** A higher-level phylogenetic classification of the Fungi. Mycological Research 111, 509-547.

- **Hibbett, D.S. y Taylor, J.W., 2013.** Fungal systematics: is a new age of enlightenment at hand? Nature Reviews Microbiology 11,129-133.

- **Huffman, J.L., Molina, F.I. y Jong, S.C., 1992.** Authentication of ATCC strains in the *Saccharomyces cerevisiae* complex by PCR fingerprinting. Experimental Mycology 16, 316-319.

- **Hull, D.L., 1997.** The ideal species concept-and why we can't get it. In: Claridge, M.F., Dawah, H.A., Wilson, M.R. (Eds.), Species: the Units of Biodiversity, Chapman & Hall, London, pp. 357-380.

- **Hurtado, A., Reguant, C., Esteve-Zarzoso, B., Bordons, A. y Rozès, N., 2008**. Microbial population dynamics during the processing of Arbequina table olives. Food Research International 41, 738-744.

- **ICMSF (Comisión Internacional de Especificaciones de Alimentos), 1996.** Microorganisms in foods 5. Microbiological specifications of food pathogens. International Commission on Microbiological Specifications for Foods. Blackie Acad, and Professional, London.

- **ICMSF (Comisión Internacional de Especificaciones de Alimentos), 1998a.** Microorganisms in foods 6. Microbial ecology of food commodities. International Commission on Microbiological Specifications for Foods. Aspen Pub. Inc. MD.

- **ICMSF (Comisión Internacional de Especificaciones de Alimentos), 1998b.** Principles of risk assessment for illness caused by foodborne biological agents. International Comision on Microbiological Specification for Foods. Journal of Food Protection 61, 1071-1074.

- **ICMSF (Comisión Internacional de Especificaciones de Alimentos), 1998c.** Potential application of risk assessment techniques to microbiological issues related to international trade in food and food products. International Comision on Microbiological Specification for Foods. Journal of Food Protection 61, 1075-1086.

- **Jacques, N., Louis-Mondesir, C., Coton, M., Coton, E. y Casaregola, S., 2014.** Two novel *Saccharomycopsis* species isolated from black olive brines and a tropical plant. Description of *Saccharomycopsis olivae* f. a., sp. nov. and *Saccharomycopsis guyanensis* f. a., sp. nov. Reassignment of *Candida amapae* to *Saccharomycopsis amapae* f. a., comb. nov., *Candida lassenensis* to *Saccharomycopsis lassenensis* f. a., comb. nov. and *Arthroascus babjevae* to *Saccharomycopsis babjevae* f. a., comb. nov. International Journal of Systematic and Evolutionary Microbiology 64, 2169-2175.

- **James, T.Y., Kauff, F., Schoch, C.L., Matheny, P.B., Hofstetter, V., Cox, C.J., Celio, G., Gueidan, C., Fraker, E., *et al*., 2006.** Reconstructing the early evolution of fungi using a six-gene phylogeny. Nature 443, 818-822.

- **Jensen, P.R. y Fenical, W., 1996.** Marine bacterial diversity as a resource for novel microbial products. Journal of Industrial Microbiology 17, 346-351.

- **Jost, L., 2007.** Partitioning diversity into independent alpha and beta components. Ecology 88, 2427-2439.

- **Juste, A., Thomma, B.P. y Lievens, B., 2008**. Recent advances in molecular techniques to study microbial communities in food associated matrices and processes. Food Microbiology 25, 745-761.

- **Kirk, P.M., *et al*., 2012**. Index Fungorum no. 2. Index Fungorum [online] http://www.indexfungorum.org/Publications/Index%20Fungorum%20no.2.pdf

- **Klein, G., 2003.** Taxonomy, ecology and antibiotic resistance of enterococci from food and the gastro-intestinal tract. International Journal of Food Microbiology 88, 123-131.

- **Konopka, A., 2006**. Microbial ecology: Searching for principles. Microbe 1, 175-179.

- **Koussémon, M., Combet-Blanc, Y., Patel, B. K., Cayol, J.L., Thomas, P., Garcia, J.L. y Ollivier, B., 2001.** *Propionibacterium microaerophilum* sp. nov., a microaerophilic bacterium isolated from olive mill wastewater. International Journal of Systematic and Evolutionary Microbiology 51, 1373-1382.

- **Kurtzman, C.P. y Robnett, C.J., 1998.** Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. Antonie van Leeuwenhoek 73, 331-371.

- **Kurtzman, C.P., Fell, J.W. y Boekhout, T., 2011a.** The Yeasts, a Taxonomic Study, 5th edn. Elsevier, Amsterdam.

- **Kurtzman, C.P., Fell, J.W., Boekhout, T. y Robert, V., 2011b.** Methods for isolation, phenotypic characterization and maintenance of yeasts. In: Kurtzman CP, Fell JW & Boekhout T (Eds) The Yeasts, a Taxonomic Study, 5th edn. Elsevier, pp 87-110.

- **Kurtzman, C.P. y Robnett, C.J., 2013.** Relationships among genera of the *Saccharomycotina* (Ascomycota) from multigene phylogenetic analysis of type species. FEMS Yeast Research 13, 23-33.

- **Kurtzman, C.P., Quintilla-Mateo, R., Kolecka, A., Theelen, B., Robert, V. y Boekhout, T., 2015.** Advances in yeast systematics and phylogeny and their use as predictors of biotechnologically important metabolic pathways. FEMS Yeast Research, 15, 1-17.

- **Lapage, S.P., Sneath, P.H.A, Skerman, V.B.D., Lessel, E.F., Seeliger, H.P.R. y Clark, W.A., 1992.** International Code of Nomenclature of Bacteria (1990 Revision). ASM, Washington, D.C.

- **León-Romero, A., Domínguez-Manzano, J., Garrido-Fernández, A., Arroyo-López, F.N., y Jiménez-Díaz, R., 2016.** Formation of In Vitro Mixed-Species Biofilms by *Lactobacillus pentosus* and Yeasts Isolated from Spanish-Style Green Table Olive Fermentations. Applied and Environmental Microbiology 82, 689-695.

- **Leroy, F. y De Vuyst, L., 2004.** Lactic acid bacteria as functional starter cultures for the food fermentation industry. Trends in Food Science & Technology 15, 67-78.

- **Liu, S.Q., 2002.** A review: malolactic fermentation in wine-beyond deacidification. Journal of Applied Microbiology 92, 589-601.

- **Lucena-Padrós, H., González, J.M., Caballero-Guerrero, B., Ruiz-Barba, J.L. y Maldonado-Barragán, A., 2014a.** *Enterococcus olivae* sp. nov., isolated from Spanish-style green-olive fermentations. International Journal of Systematic and Evolutionary Microbiology 64, 2534-2539.

- **Lucena-Padrós, H., Caballero-Guerrero, B., Maldonado-Barragán, A. y Ruiz-Barba, J.L., 2014b.** Microbial diversity and dynamics of Spanish-style green table-olive fermentations in large manufacturing companies through culture-dependent techniques. Food Microbiology 42, 154-165.

- **Lucena-Padrós, H., González, J.M., Caballero-Guerrero, B., Ruiz-Barba, J.L. y Maldonado-Barragán, A., 2014c.** *Propionibacterium olivae* sp. nov. and *Propionibacterium damnosum* sp. nov., isolated from spoiled packaged Spanish-style

green olives. International Journal of Systematic and Evolutionary Microbiology 64, 2980-2985.

- **Lucena-Padrós, H., Caballero-Guerrero, B., Maldonado-Barragán, A. y Ruiz-Barba, J.L., 2014d.** Genetic diversity and dynamics of bacterial and yeast strains associated to Spanish-style green table-olive fermentations in large manufacturing companies. International Journal of Food Microbiology 190, 72-78.

- **Lucena-Padrós, H., González, J.M., Caballero-Guerrero, B., Ruiz-Barba, J.L. y Maldonado-Barragán, A., 2015a.** *Vibrio olivae* sp. nov., isolated from Spanish-style green-olive fermentations. International Journal of Systematic and Evolutionary Microbiology 65, 1895-1901.

- **Lucena-Padrós, H., Jiménez, E., Maldonado-Barragán, A., Rodríguez, J.M. y Ruiz-Barba, J.L., 2015b.** PCR-DGGE assessment of the bacterial diversity in Spanish-style green table-olive fermentations. International Journal of Food Microbiology 205, 47-53.

- **Lucena-Padrós, H. y Ruiz-Barba, J.L., 2016.** Diversity and enumeration of halophilic and alkaliphilic bacteria in Spanish-style green table-olive fermentations. Food Microbiology 53, 53-62.

- **Ludwig, W. y Klenk, H.P., 2001.** Overview: a phylogenetic backbone and taxonomic framework for procaryotic systematics. In: Boone DR, Castenholtz RW, Garrity GM (Eds). Bergey's Manual of Systematic Bacteriology Springer: New York.

- **MAGRAMA (Ministerio de Agricultura, Alimentación y Medio Ambiente), 2014**. Aceituna de mesa. http://www.magrama.gob.es/es/agricultura/temas/ producciones-agricolas/aceite-oliva-y-aceituna-mesa/aceituna.aspx Acceso en 01.12.14

- **MAGRAMA (Ministerio de Agricultura, Alimentación y Medio Ambiente), 2015.** Diagnóstico sobre el sector de la aceituna de mesa en España. http://www.magrama.gob.es/es/agricultura/temas/producciones-agricolas/aceite-oliva-y-aceituna-mesa/aceituna.aspx Acceso en 01.03.16

- **Magurran, A.E., 2003**. Measuring biological diversity. Blackwell Publishing, Victoria, Australia.

- **Medina, E., 2008.** Antimicrobianos polifenólicos y oleosídicos en alimentos derivados de la aceituna. Tesis Doctoral, Universidad de Sevilla.

- **Millet, V. y Lonvaud-Funel, A., 2000.** The viable but non-culturable state of wine microorganisms during storage. Letters in Applied Microbiology 30, 136-141.

- **Montaño, A., Sánchez, A.H., Casado, F.J., de Castro, A. y Rejano, L., 2003.** Chemical profile of industrially fermented green olives of different varieties. Food Chemistry 82, 297-302.

- **Montaño, A., Sánchez, A.H., Casado, F.J., Beato, V.M. y de Castro, A., 2013.** Degradation of ascorbic acid and potassium sorbate by different *Lactobacillus* species isolated from packed green olives. Food Microbiology 34, 7-11.

- **Morris, C.E., Bardin, M., Berge, O., Frey-Klett, P. y Fromin, N., 2002.** Microbial biodiversity: Approaches to experimental design and hypothesis testing in primary scientific literature from 1975 to 1999. Microbiology and Molecular Biology Reviews 66, 592-616.

- **Muccilli, S., Caggia, C., Randazzo, C.L. y Restuccia, C., 2011.** Yeast dynamics during the fermentation of brined green olives treated in the field with kaolin and Bordeaux mixture to control the olive fruit fly. International Journal of Food Microbiology 148, 15-22.

- **Murray, R.G. y Schleifer, K.H., 1994.** Taxonomic notes: a proposal for recording the properties of putative taxa of procaryotes. International Journal of Systematic and Evolutionary Microbiology 44, 174-176.

- **Murray, R.G. y Stackebrandt, E., 1995.** Taxonomic note: implementation of the provisional status *Candidatus* for incompletely described procaryotes. International Journal of Systematic and Evolutionary Microbiology 45, 186-187.

- **Muyzer, G. y Smalla, K., 1998.** Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient electrophoresis (TGGE) in microbial ecology. Antonie Van Leeuwenhoek, 73, 127-141.

- **Muyzer, G., de Waal, E.C. y Uitterlinden, A.G., 1993.** Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Applied and Environmental Microbiology 59, 695-700.

- **Nisiotou, A.A., Chorianopoulos, N., Nychas, G.J.E. y Panagou, E.Z., 2010a**. Yeast heterogeneity during spontaneous fermentation of black Conservolea olives in different brine solutions. Journal Applied Microbiology 108, 396-405.

- **Nisiotou, A.A., Panagou, E.Z. y Nychas, G.J., 2010b.** *Candida olivae* sp. nov., a novel yeast species from 'Greek-style' black olive fermentation. International Journal of Systematic and Evolutionary Microbiology 60, 1219-1223.

- **Ntougias, S. y Russell, N.J., 2000**. *Bacillus* sp. WW3-SN6 a novel facultatively alkaliphilic bacterium isolated from the washwaters of edible olive. Extremophiles 4, 201-8.

- **Ntougias, S. y Russell, N.J., 2001.** *Alkalibacterium olivoapovliticus* gen. nov., sp. nov., a new obligately alkaliphilic bacterium isolated from edible-olive wash waters. International Journal of Systematic and Evolutionary Microbiology 51, 1161-1170.

- **Ntougias, S., 2012.** Alkaliphilic Lactic Acid Bacteria: Novel Sources for Genetic Engineering and Biotechnology. Gene Technology 1:e102. doi: 10.4172/2329-6682.1000e102.

- **Nychas, G.J.E., Panagou, E.Z., Parker, M.L., Waldron, K.W. y Tassou, C.C., 2002.** Microbial colonization of naturally black olives during fermentation and associated biochemical activities in the cover brine. Letters in Applied Microbiology 3, 173-177.

- **Ogier, J.C., Son, O., Gruss, A., Tailliez, P. y Delacroix-Buchet, A., 2002.** Identification of the bacterial microflora in dairy products by temporal temperature gradient gel electrophoresis. Applied and Environmental Microbiology 68(8), 3691-3701.

- **Otawa, K., Asano, R., Ohba, Y., Sasaki, T., Kawamura, E., Koyama, F., Nakamura, S. y Nakai, Y., 2006**. Molecular Analysis of Ammonia-Oxidizing

Bacteria Community in Intermittent Aeration Sequencing Batch Reactors Used for Animal Wastewater Treatment. Environmental Microbiology 8(11), 1985-1996.

- **Øvreås, L., 2000**. Population and community level approaches for analysing microbial diversity in natural environments. Ecology Letters 3, 236-251.

- **Panagou, E.Z., Schillinger, U., Franz, C.M.A.P. y Nychas, G.J.E., 2008.** Microbiological and biochemical profile of cv. Conservolea naturally black olives during controlled fermentation with selected strains of lactic acid bacteria. Food Microbiology 25, 348-358.

- **Plastourgos, S. y Vaughn, R.H., 1957**. Species of *Propionibacterium* associated with zapatera spoilage of olives. Applied Microbiology 5, 267-271.

- **Pommier, T., Pinhassi, J. y Hagstrom, A., 2005**. Biogeographic analysis of ribosomal RNA clusters from marine bacterioplankton. Aquatic Microbial Ecology 41, 79-89.

- **Powell, S.M., Riddle, M.J., Snape, I. y Stark, J.S., 2005**. Location and DGGE methodology can influence interpretation of field experimental studies on the response to hydrocarbons by Antarctic benthic microbial community. Antarctic Science 17, 353-360.

- **Prosser, J.I., Bohannan, B.J.M., Curtis, T.P., Ellis, R.J., Firestone, M.K., Freckleton, R.P., Green, J.L., Green, L.E., Killhan, K., Lennon, J.J., Osborn, A.M., Solan, M., van der Gast, C.J. y Young, P.W., 2007**. Essay- the role of ecological theory in microbial ecology. Nature Reviews Microbiology 5, 384-392.

- **Quesada, T., Aguilera, M., Morillo, J. A., Ramos-Cormenzana, A. y Monteoliva-Sánchez, M., 2007.** *Virgibacillus olivae* sp. nov., isolated from waste wash-water from processing of Spanish-style green olives. International Journal of Systematic and Evolutionary Microbiology 57, 906-910.

- **Randazzo, C.L., Torriani, S., Akkermans, A.D.L., de Vos, W.M. y Vaughan, E.E., 2002.** Diversity, dynamics, and activity of bacterial communities during production of an artisanal Sicilian cheese as evaluated by 16S rRNA analysis. Applied and Environmental Microbiology 68, 1882-1892.

- **Randazzo, C.L., Ribbera, A., Pitino, I., Romeo, F.V. y Caggia C., 2012.** Diversity of bacterial population of table olives assessed by PCR-DGGE analysis. Food Microbiology 32, 87-96.

- **Ravyts, F., Vuyst, L.D. y Leroy, F., 2012.** Bacterial diversity and functionalities in food fermentations. Engineering in Life Sciences 12, 356-367.

- **Reglamentación Técnico-Sanitaria para la Elaboración, Circulación y Venta de Aceitunas de Mesa** (Real Decreto 1230/2001). BOE nº 279, de 21 de noviembre de 2001, pp. 42587-42594.

- **Rejano, L., González-Cancho, F. y Rodríguez de la Borbolla, J.M., 1977.** Estudio sobre el aderezo de aceitunas verdes. XXIV Nuevos ensayos sobre el control de la fermentación. Grasas y Aceites 28, 255-265.

- **Rejano, L., Montaño, A., Casado, F.J., Sánchez, A.H. y de Castro, A., 2010.** Table olives: varieties and variations, in Olives and Olive Oil in Health and Disease, eds Preedy V.R., Watson R.R. editors. (Singapore: Academic Press is an imprint of Elsevier), pp 5-15.

- **Rodas, A.M., Ferrer, S. y Pardo, I., 2005.** Polyphasic study of wine *Lactobacillus* strains: taxonomic implications. International Journal of Systematic and Evolutionary Microbiology 55, 197-207.

- **Rosenzweig, M.L., 1995.** Species diversity in space and time. Cambridge University Press.

- **Rosselló-Móra, R. y Amann, R., 2001.** The species concept for prokaryotes. FEMS Microbiology Reviews 25, 39-67.

- **Rosselló-Móra, R. y Amann, R., 2015.** Past and future species definitions for Bacteria and Archaea. Systematic and Applied Microbiology 38(4), 209-216.

- **Rossetti, L. y Giraffa, G., 2005.** Rapid identification of dairy lactic acid bacteria by M13-generated, RAPD-PCR fingerprint databases. Journal of Microbiological Methods 63, 135-144.

- **Ruiz-Barba, J.L. y Jiménez-Díaz, R., 1989.** Actividad antibacteriana de las salmueras de aceitunas verdes sobre *Lactobacillus plantarum*. Grasas y Aceites 40, 102-108.

- **Ruiz-Barba, J.L., Rios-Sanchez, R.M., Fedriani-Iriso, C., Olias, J.M., Rios, J.L. y Jimenez-Diaz, R., 1990.** Bactericidal effect of phenolic compounds from green olives on *Lactobacillus plantarum.* Systematic and Applied Microbiology 13, 199-205.

- **Ruiz-Barba, J.L., Garrido-Fernández, A. y Jiménez-Díaz, R., 1991a.** Bactericidal action of oleuropein extracted from green olives against *Lactobacillus plantarum*. Letters in Applied Microbiology 12, 65-68.

- **Ruiz-Barba, J.L., 1991b.** Estudio de algunos factores que influyen en el desarrollo de una fermentación ácido-láctica apropiada de aceitunas de mesa. Tesis Doctoral, Universidad de Sevilla.

- **Ruiz-Barba, J.L., Brenes-Balbuena, M., Jiménez-Díaz, R., García-García, P. y Garrido-Fernández, A., 1993.** Inhibition of *Lactobacillus plantarum* by polyphenols extracted from two different kinds of olive brines. Journal of Applied Bacteriology 74, 15-19.

- **Ruiz-Barba, J.L., Cathcart, D.P., Warner, P.J. y Jiménez-Díaz, R., 1994.** Use of *Lactobacillus plantarum* LPCO10, a bacteriocin producer, as a starter culture in Spanish-style green olive fermentations. Applied and Environmental Microbiology 60, 2059-2064.

- **Ruiz-Barba, J.L., Caballero-Guerrero, B., Maldonado-Barragán, A. y Jiménez-Díaz, R., 2010**. Coculture with specific inducing bacteria notably enhances survival of the autoinducer-regulated bacteriocin producer *Lactobacillus plantarum* NC8 in olive fermentations. Food Microbiology 27, 413-417.

- **Ruiz-Barba, J.L. y Jiménez-Díaz, R., 2012.** A novel *Lactobacillus pentosus*-paired starter culture for Spanish-style green olive fermentation. Food Microbiology 30, 253-259.

- **Ruiz Cruz, J. y González Cancho, F., 1969.** Metabolismo de levaduras aisladas de salmueras de aceitunas aderezadas "estilo español". Grasas y Aceites, 20, 6-11.

- **Ryan, D. y Robards, K., 1998.** Phenolic Compounds in Olives. The Analyst. 123, 31-44.

- **Santos-Siles, F.J., 1999.** New technologies in table olive processing. Grasas y Aceites 50(2), 131-140.

- **Schoch, C.L., Seifert, K.A., Huhndorf, S., Robert, V., Spouge, J.L., Levesque, C.A., Chen, W., *et al*., 2012.** Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for fungi. Proceedings of the National Academy of Sciences of the United States of America 109, 6241-6246.

- **Schwartz, D.C. y Cantor, C.R., 1984.** Separation of yeast chromosome sized DNAs by pulsed field gradient gel electrophoresis. Cell 37, 67-75.

- **Scorzetti, G., Fell, J.W., Fonseca, A. y Statzell-Tallman, A., 2002.** Systematics of basidiomycetous yeasts: a comparison of large subunit D_1/D_2 and internal transcribed spacer rDNA regions. FEMS Yeast Research 2, 495-517.

- **Sekiguchi, H., Tomioka, N., Nakahara, T. y Uchiyama, H., 2001.** A single band does not always represent single bacterial strains in denaturing gradient gel electrophoresis analysis. Biotechnology Letters 23, 1205-1208.

- **Seseña, S., Sánchez, I. y Palop, M.Ll., 2005**. Characterization of *Lactobacillus* strains and monitoring by RAPD-PCR in controlled fermentations of "Almagro" eggplants. International Journal of Food Microbiology 104, 325-335.

- **Sheffield, V.C., Cox, D.R. y Myers, R.M., 1989**. Attachment of a 40- bp G+C rich sequence (GC-clamp) to genomic DNA fragments by polymerase chain reaction results in improved detection of single-base changes. Proceedings of the National Academy of Sciences of the United States of America 86, 232-236.

- **Sneath, P.H.A. y Sokal R.R., 1973.** Numerical taxonomy: The principles and practice of numerical classification. San Francisco: W. H. Freeman.

- **Sorokin, I.D., Zadorina, E.V., Kravchenko, I.K., Boulygina, E.S., Tourova, T. P. y Sorokin, D.Y., 2008.** *Natronobacillus azotifigens* gen. nov., sp. nov., an anaerobic diazotrophic haloalkaliphile from sodarich habitats. Extremophiles 12, 819-827.

- **Stackebrandt, E. y Ebers, J., 2006**. Taxonomic parameters revisited: tarnished gold standards. Microbiology Today 33, 152-155.

- **Stackebrandt, E. y Goebel, B.M., 1994**. Taxonomic Note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. International Journal of Systematic and Evolutionary Microbiology 44, 846-849.

- **Stierle, A.A., Stierle, D.B. y Kemp, K., 2004**. Novel sesquiterpenoids with matrix metalloproteinase-3 inhibitory activity from an acid mine waste extremophile. Journal of Natural Products 67, 1392-1395.

- **Stierle, A.A., Stierle, D.B. y Kelly, K., 2006.** Berkelic acid, a novel spiroketal with selective anticancer activity from an acid mine waste fungal extremophile. Journal of Organic Chemistry 71, 5357-5360.

- **Sugita, T., Nakajima, M., Ikeda, R., Matsushima, T. y Shinoda, T., 2002.** Sequence analysis of the ribosomal DNA intergenic spacer 1 regions of *Trichosporon* species. Journal of Clinical Microbiology 40, 1826-1830.

- **Sun, H.Y., Deng, S.P. y Raun, W.R., 2004.** Bacterial Community Structure and Diversity in a Century-Old Manure-Treated Agroecosystem. Applied and Environmental Microbiology 70, 5868-5874.

- **Sutcliffe, I.C., Trujillo, M.E. y Goodfellow, M., 2012.** A call to arms for systematists: revitalising the purpose and practises underpinning the description of novel microbial taxa. Antonie Van Leeuwenhoek 101, 13-20.

- **Tailliez, P., Quénée, P. y Chopin, A., 1996.** Estimation de la diversité parmi les souches de la collection CNRZ: application de la RAPD à un groupe de lactobacilles. Lait 76, 147-158.

- **Takemura, A.F., Chien, D.M. y Polz, M.F., 2014.** Associations and dynamics of *Vibrionaceae* in the environment, from the genus to the population level. Frontiers in Microbiology 5:38. doi: 10.3389/fmicb.2014.00038.

- **Taylor, J.W., Jacobson, D.J., Kroken, S., Kasuga, T., Geiser, D.M., Hibbett, D.S. y Fisher, M.C., 2000.** Phylogenetic species recognition and species concepts in fungi. Fungal Genetics and Biology 31, 21-32.

- **Thompson, F.L., Iida, T. y Swings, J., 2004.** Biodiversity of vibrios. Microbiology and Molecular Biology Reviews 68, 403-431.

- **Tindall, B.J., Rosselló-Móra, R., Busse, J.H., Ludwig, W. y Kämpfer, P., 2010**. Notes on the characterization of prokaryote strains for taxonomic purposes. International Journal of Systematic and Evolutionary Microbiology 60, 249-266.

- T**ofalo, R., Perpetuini, G., Schirone, M., Suzzi, G. y Corsetti, A., 2013.** Yeast biota associated to naturally fermented table olives from different Italian cultivars. International Journal of Food Microbiology 161, 203-208.

- **Tofalo, R., Perpetuini, G., Schirone, M., Fasoli, G., Suzzi, G. y Corsetti, A., 2014.** *Lactobacillus pentosus* dominates spontaneous fermentation of Italian table olives. LWT-Food Science and Technology 57, 710-717.

- **Torriani, S., Felis, G.E. y Dellaglio, F., 2001.** Differentiation of *Lactobacillus plantarum, L. pentosus,* and *L. paraplantarum* by *recA* Gene Sequence Analysis and Multiplex PCR Assay with *recA* Gene-Derived Primers. Applied and Environmental Microbiology 67, 3450-3454.

- **Torsvik, V., Daae, F.L., Sandaa, R.A. y Øvreås, L., 1998.** Review article: novel techniques for analysing microbial diversity in natural and perturbed environments. Journal of Biotechnology 64, 53-62.

- **Tristezza, M., Vetrano, C., Bleve, G., Grieco, F., Tufariello, M., Quarta, A., Mita, G., Spano, G. y Grieco, F., 2011.** Autochthonous fermentation starters for the industrial production of Negroamaro wines. Journal of. Industrial Microbiology & Biotechnology 39, 81-92.

- **Valmorri, S., Tofalo, R., Settanni, L., Corsetti, A. y Suzzi, G., 2010.** Yeast microbiota associated with spontaneous sourdough fermentations in the production of traditional wheat sourdough breads of the Abruzzo region (Italy). Antonie Van Leeuwenhoek 97, 119-129.

- **Vandamme, P., Pot, B., Gillis, M., de Vos, P., Kersters, K. y Swings, J., 1996.** Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiological Reviews 60, 407-438.

- **Vaughn, R.H., Douglas H.C. y Gillilandj, R., 1943.** Production of Spanish-style green olives. California Agricultural Experiment Station Publications 678.

- **Versalovic, J., Koeuth, T. y Lupski, J.R., 1991.** Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Research 19, 6823-6831.

- **Vos, M., 2011.** A species concept for bacteria based on adaptive divergence. Trends Microbiology 19, 1-7.

- **Ward, D.M., 1998.** A natural species concept for prokaryotes. Current Opinion in Microbiology 1, 271-277.

- **Wayne, L.G., Brenner, D.J., Colwell, R.R., Grimont, P.A.D., Kandler, O., Krichevsky, M.I., Moore, L.H., Moore, W.E.C., Murray, R.G.E., Stackebrandt, E., Starr, M.P. y Trüper, H.G., 1987**. Report of the ad hoc Committee on Reconciliation of Approaches to Bacterial Systematics. International Journal of Systematic and Evolutionary Microbiology 37, 463-464.

- **Whittaker, R.H., 1977**. Evolution of species diversity in land communities. Evolutionary Biology 10, 1-67.

- **Williams, J.G., Kubelik, A.R., Livak, K.J., Rafalski, J.A. y Tingey, S.V., 1990.** DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research 18, 6531-6535.

- **Woese, C.R., Kandler, O. y Wheelis, M.L., 1990**. Towards a natural system of organisms: Proposal for the domains Archaea, Bacteria, and Eucarya. Proceedings of the National Academy of Sciences of the United States of America 87, 4576-4579.

- **Yarrow, D., 1998.** Methods for the isolation, maintenance and identification of yeasts. In: Kurtzman C.P. y Fell J.W. (Eds). The Yeasts. A Taxonomic Study, 4th edn. Elsevier, 418 Amsterdam, pp 77-100.

- **Yooseph, S., Nealson, K.H., Rusch, D.B., McCrow, J.P., Dupont, C.L., Kim, M., *et al*., 2010**. Genomic and functional adaptation in surface ocean planktonic prokaryotes. Nature 468, 60-66.

- **Young, J.M., Crawford, J.W., Nunan, N., Otten, W. y Spiers, A., 2008**. Microbial distribution in soils: Physics and scaling. Advances in Agronomy 100, 81-121.

- **Zettler, E.R., Mincer, T.J. y Amaral-Zettler, L.A., 2013**. Life in the "Plastisphere": microbial communities on plastic marine debris. Environmental Science & Technology 47, 7137-7146.

- **Zhi, X.Y., Zhao, W., Li, W.J. y Zhao, G.P., 2012.** Prokaryotic systematics in the genomics era. Antonie van Leeuwenhoek 101, 21-34.

- **Zhilina, T.N., Garnova, E.S., Tourova, T.P., Kostrikina, N.A. y Zavarzin, G.A., 2001**. *Amphibacillus fermentum* sp. nov. and *Amphibacillus tropicus* sp. nov., new alkaliphilic, facultatively anaerobic, saccharolytic bacilli from Lake Magadi. Microbiology 70, 711-722.

- **Zinger, L., Gobet, A. y Pommier, T., 2012.** Two decades of describing the unseen majority of aquatic microbial diversity. Molecular Ecology 21, 1878-1896.

- **Zohary, D. y Spiegel-Roy, P., 1975**. Beginning of fruit growing in the old world. Science 187, 319-327.